Characterisation of mechanisms of insecticide resistance in Malaysian populations of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

Submitted by

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DECLARATION

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree.

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DEDICATION

Especially to my husband, Shamshul Ahmad Shah, my parents, Hasnah Haron and Ishak Ismail, my dearest siblings, Intan Hafizah, Khairon Ariffin and Sara Ruqqaiyah. With my deepest appreciation for their love, patience and encouragement. I love you all.

ABSTRACT

Aedes aegypti and *Aedes albopictus* are vectors of dengue fever in Malaysia. Vector control is the only means for the prevention of this disease. Unfortunately, insecticide resistance in this mosquito is threatening vector control programs. The present study intended to fill important knowledge gaps on the extent, geographical distribution and underlying mechanisms of these resistances in Malaysia.

Mosquitoes were collected from four states in Malaysia in 2010 to assess their resistance status. WHO bioassays showed multiple resistance profiles in all populations. Multiple and high levels of resistance was observed for *Ae. aegypti* particularly in Kuala Lumpur, while resistance was more moderate in *Ae. albopictus*. PBO synergist assays indicated the presence of multiple resistance mechanisms for *Ae. aegypti* whereas it suggested a predominance of metabolic resistance through cytochrome P450s for *Ae. albopictus* populations.

Investigation of resistance mechanisms in *Ae. aegypti* populations detected the F1534C *knockdown resistance (kdr)* mutation using pyrosequencing in all four populations with frequency ranging from 40% to 80%. However, apart from Penang and Johor Bharu, a correlation was not always found with resistance phenotypes. Furthermore, the sequencing of a cDNA fragment spanning exons 19 to 31 of the voltage gated sodium channel (VGSC),detected the V1016G *kdr* mutation in the four populations although at a lower frequency than F1534C. No correlation was observed between V1016G and resistance phenotype. Genome-wide transcription analysis using microarray detected the genes associated with metabolic resistance in these populations. Several cytochrome P450 genes (CYP9J27, CYP6CB1, CYP9J26 and CYP9M4) were among the most up-regulated genes in all populations indicating that they may play an important role in the observed resistance. This list also included glutathione-S-transferases, carboxylesterases and other gene families commonly associated with insecticide. Quantitative real-time PCR (qRT-PCR) validated the over-expression of all cytochrome P450s except for CYP6CB1.

Analysis of resistance mechanisms in *Ae. albopictus* revealed significant differences to those observed in *Ae. aegypti*. Firstly, no *kdr* mutation was detected in this species across Malaysia. The absence of *kdr* mutation was further strengthened by the high genetic diversity of a portion of this gene. Secondly, analysis of the genome-wide transcription profile of *Ae. albopictus* populations revealed that cytochrome P450s from the CYP6 family are playing a predominant role in the observed resistance in contrast to CYP9 family for *Ae. aegypti*. Microarray analysis using a new 8 x 60k Agilent *Ae. albopictus* chip detected 40 genes commonly over-expressed including the cytochrome P450 genes CYP6N9, CYP9AE1 and CYP6AG6. A direct comparison of permethrin-resistant against non-exposed Kuala Lumpur mosquitoes revealed that permethrin resistance was most likely conferred by a combination of a reduced cuticle penetration and detoxification through cytochrome P450s. Indeed, several genes from these two gene families were consistently highly over-expressed. P450 transcripts corresponding to CYP6P4 in *An. gambiae* or CYP6P12 in *Ae. aegypti* were consistently the most up-regulated P450. Other up-regulated genes included GST, ABC transporters, heat shock protein, oxidaxes, and proteases. qRT-PCR analysis validated these expression patterns with the P450 CYP6N3 the most over-expressed detoxification gene.

This study revealed significant differences in the resistance profile and the underlying resistance mechanism between the two dengue vectors across Malaysia. This presents a challenge for the common control of both species as it appears that a single insecticide-based control intervention may not efficiently control both species.

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Ae.	*Aedes*
AChE	acetylcholinesterase
An.	*Anopheles*
Bti	*Bacillus thuringiensis israelensis*
BLAST	basic local alignment and search tool
Bs	*Bacillus sphaericus*
cDNA	complimentary DNA
Cx.	*Culex*
D.	*Drosophila*
DDT	dichlorodiphenyltrichoroethylene
DF	dengue fever
DHF	dengue haemorrhagic fever
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
GABA	gamma-aminobutyric acid
gDNA	genomic DNA
GST	glutathione-S transferase
IRS	indoor residual spraying
ITN	insecticide treated net
JB	Johor Bharu
KB	Kota Bharu
kdr	knockdown resistance
KL	Kuala Lumpur
LC_{50}	median lethal concentration
LLIN	long lasting insecticidal nets
LSTM	Liverpool School of Tropical Medicine
M	molar
$MgCl_2$	magnesium chloride
Min	minutes
NaCl	sodium chloride
NO	New Orleans

OP	organophosphate
PBO	piperonyl butoxide
PCR	polymerase chain reaction
PG	Penang
PY	pyrethroid
P450	cytochrome P450
qRT-PCR	quantitative real-time polymerase chain reaction
Rdl	resistance to dieldrin
RFLP	restriction fragment length polymorphism
RIDL	release of insects with a dominant lethal
RNA	ribonucleic acid
RR	resistance ratio
SIT	sterile insect technique
SNP	short nucleotide polymorphism
ULV	ultra low volume
USM	University Sains Malaysia
VCRU	Vector Control Research Unit
W.	*Wolbachia*
WHO	World Health Organisation
WHOPES	WHO pesticide evaluation scheme

1.0 LITERATURE REVIEW

1.1 Introduction

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are the most rapidly spreading vector-borne diseases with approximately 50 million cases of infection worldwide (WHO, 2012a). However, a recent study using modelling and cartographic approaches has shown that the number of dengue cases could be two times higher (96 million cases) than what is reported by WHO (Bhatt *et al.*, 2013). Dengue mainly occurs in tropical and subtropical areas around the globe. Currently, the Americas, Southeast Asia and the Western Pacific regions are the most affected regions with cases exceeding 2.3 million in 2010 (WHO, 2012b). Malaysia is one of the affected countries in Southeast Asia. Dengue cases in Malaysia were 27.5 cases/100,000 population in 1990 and increased drastically to 123.4 cases/100,000 population in 1998 during the global pandemic (Ang and Satwant, 2001). In 2010, the number of cases increased to 46,171 cases which is 1648.96 cases/100,000 population with 134 deaths (MOH, 2011).

DF and DHF are caused by the dengue virus, which belongs to the genus *Flavivirus*, family Flaviviridae, that consists of 4 dengue virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) (Miyagi and Toma, 2000). Once infected by one serotype, a person is immune to that particular serotype. However, cross-immunity to the other serotypes after recovery is temporary and subsequent infections by other serotypes increase the risk of developing DHF or severe dengue (WHO, 2012b). Dengue viruses are transmitted from viremic to susceptible human beings by various mosquitoes of the subgenus *Stegomyia*, notably *Aedes aegypti* and *Aedes albopictus* (Miyagi and Toma, 2000).

In 2013, a fifth dengue serotype was discovered. Samples collected from Sarawak (East Malaysia) showed a phylogenetically different dengue virus serotype from the previous four serotypes (Abu Hassan and Yap, 1999, Normile, 2013). However, the fifth serotype belongs to a sylvatic cycle which only circulates in non-human primates (Normile, 2013). Even though the fifth

serotype has not caused dengue fever outbreaks, the discovery of this serotype could complicate vaccine development.

There are approximately 500 *Aedes* species mosquito in the world (Arbain, 1990, Abu Hassan and Yap, 1999). Some of the *Aedes* species are closely associated with humans, especially *Ae. aegypti* and *Ae. albopictus,* because of their ability to breed in the areas of human dwellings either in towns or in rural areas (Klowden, 1993). This close relationship is causing concern since their blood feeding behaviour is the cause of disease transmission (Arbain, 1990).

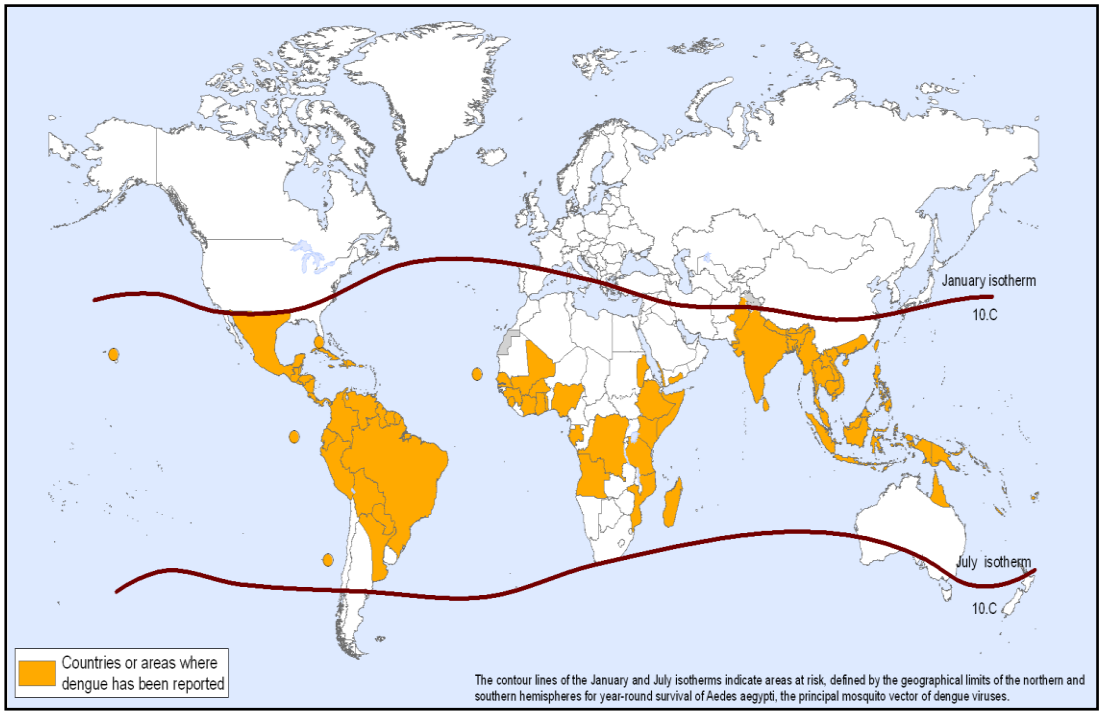


Figure 1.1 Map of global dengue distribution. Taken from WHO (2011).

The observations over 100 years have shown that the epidemiology of dengue varies a great deal with respect to both geography and time. This is due not only to modifications in human ecology (population increase, urbanization, more frequent travel), but also to ecological adaptations of certain mosquito species (Ang and Satwant, 2001). In Malaysia, over the last two decades, rapid changes in the urban environment and demographic structure in the country has undoubtedly influenced changes in the vector ecology and consequently the epidemiology of dengue.

Dengue control relies exclusively on vector control to eliminate the vector mosquitoes as there are no anti-virals or vaccines. Sanitation and elimination of breeding sites are the major method of mosquito control for the long term (Horstick *et al*., 2010). However, this approach has been proven difficult without the collaboration of local people (Goma, 1966, Rohani et al., 2011, Pilger et al., 2009).

Chemical control interventions through the use of insecticide are becoming increasingly common. These insecticide-based interventions include: household aerosol, mosquito coil, mat and vaporized liquid (Lee and Yap, 2003). Insecticide impregnated materials such as nets and curtains are also abundant using mainly the pyrethroid class (Lenhart et al., 2008, McCall and Lenhart, 2008). In addition, insecticides could be applied using thermal spraying, Ultra Low Volume (ULV) spraying and surface residual spray to kill adult mosquitoes (Malaysia, 2008). Chemicals are also used for larviciding such as the application of Abate® that targets the immature stage (Chen *et al*., 2008b).

Over dependence on chemical insecticides, has led to increasing cases of insecticide resistance in *Aedes* populations worldwide (Hemingway and Ranson, 2000, Ranson et al., 2008) threatening the continued success of current vector control interventions. In Malaysia, evidence of resistance towards permethrin and temephos has been recorded in both vectors of dengue in Kuala Lumpur and Penang (Nazni et al., 2009, Chan et al., 2011). However, most studies mainly focus on these two big cities in Malaysia and little is known about the resistance profile of *Ae. aegypti* and *Ae. albopictus* across Malaysia. Such information is needed in order to design and implement suitable control interventions against these species.

This study was conducted to fill this knowledge gap by investigating the resistance profiles of these two important arbovirus vectors to several classes of insecticides and elucidating the underlying resistance mechanisms.

1.2 Mosquito classification

Mosquitoes have a worldwide distribution, from tropical areas, subtropical areas and the Arctic (Service, 2012). There are 3100 species of mosquitoes comprising of 34 genera and divided into three subfamilies: Toxorhynchitinae, Culicinae and Anophelinae.

Mosquito classification according to Knight and Stone (1977) is as follows.

Class	: Insecta	
Order	: Diptera	
Family	: Culicidae	
Subfamily	: Toxorhynchitinae	Genus: *Toxorhynchitinae*
	Culicinae	*Aedes*, *Culex, Mansonia*
	Anophelinae	*Anopheles*

Mosquitoes are vectors of several diseases to human such as malaria (protozoa), filariasis (nematode) and viral diseases (dengue, yellow fever, chikungunya and encephalitis) (Miyagi and Toma, 2000). Only mosquitoes from the genera *Anopheles,* Aedes*, Culex* and *Mansonia* have medical importance (Harwood and James, 1979, Service, 2012, Abu Hassan and Yap, 1999).

This study focuses on *Ae. aegypti* and *Ae. albopictus* because they are the main vectors of dengue and dengue haemorrhagic fever and chikungunya in Malaysia. The incidence of these diseases is increasing resulting in the loss of many lives (MOH, 2011).

1.3 Bionomics of *Aedes aegypti* and *Aedes albopictus*

1.3.1 *Aedes* distribution

The *Aedes* genus comprises of 500 species, but not all are of medical importance (Abu Hassan and Yap, 1999). *Aedes aegypti* and *Ae. albopictus*

population distribution are growing due to their ability to breed in containers found in human settlements such as residential areas and shop lots (Klowden, 1993, Rohani *et al.*, 2011, Promprous *et al.*, 2005). *Aedes aegypti* lays their eggs in man-made containers while *Ae. albopictus* breeds in natural pools of standing water; in the leaves and in tree holes (Hundson *et al.*, 1998, Lee and Yap, 2003). There is evidence that shows both *Aedes* species could breed in the same container, either man-made or natural breeding sites. *Aedes aegypti* and *Ae. albopictus* mixed breeding was found in water containers surveyed and occurred mainly in outdoor containers of large size (Chen *et al.*, 2006). Chen and colleagues (2006) reported that more *Ae. aegypti* could be observed breeding both indoors and outdoors compared to *Ae. albopictus* which was mainly found outdoors.

Aedes albopictus is an indigenous species in Malaysia. It is believed that this mosquito originated in the tropical forest of Southeast Asia (Smith, 1956). *Aedes aegypti* is believed to originate from the tropical rainforests in Africa (Vythilingam *et al.*, 1992) and the invasion of this species into Malaysia was first recorded by Leicester in 1908 and Stanten in 1914 (Lee and Cheong, 1987). The distribution of *Ae. aegypti* and *Ae. albopictus* in Peninsular Malaysia and East Malaysia has been well established (Hii, 1977, Lee, 1991).

Aedes aegypti dispersed from Africa throughout the tropics during the height of the slave trade in the 16th and 17th century (Kaplan *et al.*, 2010). The increase in global transportation as well as trading was the likely reason that brought this species into Malaysia. *Aedes aegypti* could also be found to be well established in United States, Latin America, Europe and Asia (Service, 2012, Vontas et al., 2012).

Aedes albopictus was most likely brought into the United States through tyre trades (Paupy *et al.*, 2009). The eggs of this mosquito species were stuck in the tires which was one of their breeding sites and exported from Asia to the United States (Reiter and Sprenger, 1987). This was also the cause of spreading to countries such as Brazil, Mexico, Dominican Republic, Nigeria, Fiji, Albania and Italy (Delatte *et al*., 2008). Recently, *Ae. albopictus* could be

found in Croatia, Greece, France, Netherlands, Spain and Switzerland (Paupy *et al.*, 2009).

1.3.2 Morphology of *Aedes aegypti* and *Aedes albopictus*

Both *Aedes* species are small in size measuring from 4 to 6mm, have a pair of wings and a pair of halters on their back and three pairs of legs with white rings (Clements, 1992). Virtually all Aedes mosquitoes are black with white stripes, especially on the thorax. *Aedes aegypti* can be identified by having two curved lines on the sides and a straight line in the middle of its thorax (Figure 1.2). *Aedes albopictus* has only one straight line in the middle of its thorax (Figure 1.3).

Figure 1.2 Patterns on the thorax of *Aedes aegypti*
(*Source: http://en.ird.fr/the-media-centre/scientific-newssheets)*

Figure 1.3 Patterns on the thorax of *Aedes albopictus*
(*Source: http://demamchikungunya.blogspot.co.uk)*

1.4 Biology and life cycle of *Aedes*

Aedes undergo a holometabolous life cycle which is a complete metamorphosis; through the eggs, larvae, pupae and adults (Goma, 1966). The life cycle of the *Aedes* mosquito from eggs to adult usually takes 10-12 days (Service, 2012). Female mosquitoes need to take a blood meal and have to mate only once in their lifetime to lay eggs. The process of taking

a blood meal, digesting the blood for the formation of eggs and laying their eggs is called gonotrophic cycle (Abu Hassan and Yap, 1999).

Aedes eggs are black, oval and are unattached to each other. *Aedes* mosquitoes will lay eggs in moist substrates such as on the water surface on the edge/wall of the water cans, flower pots and tree holes (Service, 2012, Clements, 1992). The eggs have an elastic protein wall to reduce water loss and allow gas exchange (Nasci and Miller, 1996). *Aedes* eggs can withstand drying and can still hatch even in prolonged dry conditions. Once exposed to water, the embryos in the eggs will expand. It will hatch when there is a stimulus such as a lack of oxygen in the water, the change in length of day and temperature (Clements, 1992).

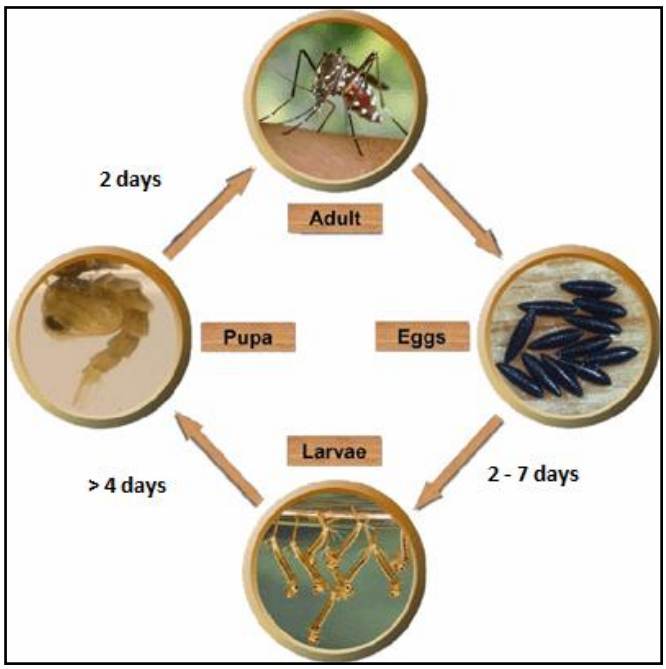


Figure 1.4 Life cycle of Aedes mosquito
(Source: http://sigmabiotech.com/mosquitoes.html)

The eggs will hatch through several stages, meaning that not all eggs will hatch at the same time. This creates problems in the measures to control mosquito larvae (Nasci and Miller, 1996, Service, 2012). Due to the different hatching rates of the eggs, during a control treatment some larvae that have been exposed might be killed by the larvicides but the embryo in the

unhatched eggs are still viable and would not be killed in this treatment period (Abu Hassan and Yap, 1999).

Aedes mosquito larvae can be found in containers of clean and stagnant water (Abu Hassan and Yap, 1999). Mosquitoes go through four stages of aquatic larvae. They usually feed on microorganisms, detritus, algae and other organic matter such as dead invertebrates (Nasci and Miller, 1996, Service, 2012, Clements, 1992). The feeding mode of Aedes larvae is collecting-gathering which involves first causing organic materials that have settled or attached to surfaces to resuspend and then ingesting the mixture that has been resuspended (Clements, 1992).

Mosquito pupae are also aquatic organisms that are active and motile. The head and thorax of the pupae are joined as a cephalothorax and have two respiratory trumpets. This pupae stage does not feed (Nasci and Miller, 1996). Pupae have all the adult organs but are not fully developed (Goma, 1966). When the metamorphosis is complete and adult mosquitoes are fully developed in the pupae cuticle, the pupae will suck in the air from the atmosphere to increase the internal pressure and the cuticle will break allowing the adult mosquitoes to become free (Nasci and Miller, 1996).

After emerging from the shells of pupae (chrysalis), the soft cuticle of the adult mosquito will harden and the mosquito will be able to fly within 10-15 minutes. Adult mosquitoes are divided into three parts. On the head, there is a pair of compound eyes, a pair of antenna, a pair of long palpus and proboscis (Nasci and Miller, 1996, Service, 2012). On the thorax there is a pair of wings, halteres and three pairs of tapered legs. The abdomen consists of 10 segments in which the two segments are modified for reproductive purposes (Clements, 1992). The mosquito body is covered with fine hair and scales in a certain pattern. The patterns are important to identify the species of mosquito (Nasci and Miller, 1996, Service, 2012).

The antenna on male mosquitoes is feather-like (plumose) and the end of its palpus is the same length as its proboscis. For female mosquitoes, the antenna is filament-like and the palpus is shorter than the proboscis (Nasci and Miller, 1996). Once able to fly, adult mosquitoes will find a place to take

shelter and are ready to mate. For female mosquitoes, they can mate a few hours after emerging from the pupa, but the male mosquito will not be able to copulate until their genitalia has turned 180° to the position that it should be roughly around 20-24 hours (Service, 2012).

Both adult male and female mosquitoes feed on plant sugars as an energy source for biological activity, metabolic functions and also to fly (Foster, 1995, Magnarelli *et al.*, 1979, Nasci and Miller, 1996). Plant sugar is the major food source and comes in the form of floral nectar, vegetative tissue and damaged fruit. Blood meals are only taken by females as a source of protein which is needed for ovary development (Clements, 1992, Service, 2012). Females usually take as much as 2-4 times their weight of blood in a single meal and will excrete fluid while bloodfeeding to concentrate the protein. Blood meal will be taken after mating (Service, 2012).

1.5 The behaviour and characteristics of *Aedes*

For *Aedes* mosquito, mating occurs in the air. Instead of forming swarms, the male *Aedes* tend to aggregate around a host which is a primary female encounter site (Ponlawat and Harrington, 2009). Males will grasp females in flight as they approach or leave hosts after blood feeding. The sound produced by the wings of female mosquitoes while flying will attract male mosquitoes (Service, 2012, Clements, 1992). The male mosquitoes detect the females using the Johnston organ on its antenna (Nasci and Miller, 1996).

There are certain species of *Aedes* mosquito that prefers to feed on human blood (anthropophilic) and some prefer feeding on blood from animals (zoophilic) (Clements, 2012). There are two factors driving the female mosquitoes to choose their preferred host, intrinsic factors and extrinsic factors. Intrinsic factors are internal factors such as the desire to suck blood to lay their eggs and other genetic elements. Extrinsic factors are the external factors such as temperature, heat, humidity and carbon dioxide emitted by the host (Arbain, 1990).

Aedes aegypti mosquitoes prefer to bloodfeed indoors (termed as endophagic) and prefer to rest in dark places inside the house (endophilic). They primarily feed during the day and are most active two hours after dawn and a few hours before dusk (Sulaiman, 1990, CDC, 2012a). Flight range studies suggests that most *Ae. aegypti* mosquitoes spend their lifetime around houses where they emerge as adults and they usually fly an average of 400 meters (CDC, 2012a).

The highlight of *Ae. albopictus* biting activity is during early morning and late afternoon and they are a very aggressive daytime biter (Lee, 2000). *Aedes albopictus* bites humans indoors and outdoors (endophagic and exophagic) but they prefer to feed inside homes (Reid, 1961). They also like to rest on the vegetation (exophilic) (Lee, 2000, Chen et al., 2006). *Aedes albopictus* have a short flight range which is less than 200 meters (CDC, 2012b). The eggs of *Ae. albopictus* remains viable in the winter in temperate climates and are active throught the year in countries with tropical weather (Clements, 1992, CDC, 2012b)

The frequency of blood feeding for both species depends on the time required to complete a gonotrophic cycle which usually takes 3-5 days (Becker, 2010). The more frequent blood feeding occurs, the probability of transmitting diseases are higher (Service, 2012).

1.6 The role of *Aedes* mosquitoes as vectors of disease

The important characteristics of a mosquito that allows it to transmit diseases to humans are: their attraction to human host for blood meal, their persistence in the environment, and their longevity, allows the disease-causing organisms to grow to the infective stage, and their capacity for repeated blood feeding (Goma, 1966, Rohani et al., 2011).

Dengue fever, yellow fever, chikungunya and encephalitis are caused by arboviruses. Arboviruses are viral agents that replicate in arthropods and are transmitted by arthropods. Most arboviruses do not infect humans because they cannot replicate in humans or because the vectors do not come into

contact with humans. Due to the advancement of technology, human encroachment into the remote areas (the rain forest and the tundra) there is an increase probability of human-vector convergence. The introduction of exotic mosquitoes as vectors in the new habitat increases the chances of humans being infected with arbovirus pathogens and becoming viraemic (Nasci and Miller, 1996, Service, 2012).

Usually, *Ae. aegypti* are primary vectors of DF, DHF and also chikungunya but in locations where *Ae. aegypti* are absent or rare, *Ae. albopictus* have been the major vector (Delatte *et al.*, 2010). *Aedes albopictus* have become the major dengue vector in Hawaii (Delatte *et al.*, 2010) and the spread of chikungunya in French island of Reunion in (WHO, 2008a).

1.6.1 The vectors of dengue fever and dengue haemorrhagic fever

Aedes aegypti and *Ae. albopictus* are vectors of dengue fever (DF) and dengue haemorrhagic fever (DHF). DF and DHF are caused by the dengue virus of the Flavivirus genus which contains four serotypes called DEN-1, DEN-2, DEN-3 and DEN-4. It is the most important vector borne disease caused by viruses and the number of infection cases is increasing throughout the world (Service, 2012, Miyagi and Toma, 2000, WHO, 2012b, Bhatt *et al.*, 2013).

1.6.1.1 Epidemiology of dengue fever and dengue haemorrhagic fever

Dengue fever is endemic in the tropics, especially in Asia, the Pacific, Africa and America. Dengue hemorrhagic fever usually occurs in urban areas in Southeast Asia and India. DHF's first epidemic case was first reported in the 1950s in the Philippines and Thailand (WHO, 2012b) and also Cuba in 1981 with 158 cases of mortality. There is evidence that shows dengue originated from tropical Asia and spread to Africa (Service, 2012). *Aedes aegypti* is a peridomestic mosquito and its anthropophilic behaviour is the reason that it is the main vector for all serotypes of dengue virus. All areas with *Ae. aegypti*

are potentially at risk of dengue fever. The presence of *Ae. albopictus* together with *Ae. aegypti* further increases the risk of dengue due to both species are able to transmit the dengue virus (Lambrechts et al., 2010). However, *Ae. albopictus* plays a minor role compared to *Ae. aegypti* in dengue transmission due to the difference in host preference and vector competence (Lambrechts *et al.*, 2010).

There are no reservoir host that can be identified for the dengue virus (Service, 2012). However, according to Knudsen *et al.* (1977) and Wallace *et al*. (1980), there is a cycle of dengue in the Malaysian jungle involving monkeys that live in the forest canopy and forest mosquito species, *Aedes niveus* that blood feeds on humans and monkeys. There is also a sylvatic cycle of DENV-2 virus in Eastern Senegal (Vasilakis and Weaver, 2008).

1.6.1.2 Symptoms of infection and the status of dengue fever and dengue haemorrhagic fever

Dengue fever, also known 'break bone fever' is not fatal. Signs of infection are sudden fever, severe headache, back pain and intense pain in the joints (Goma, 1966). Primary infections can result in a variety of symptoms, such as fever and headache, or be asymptomatic. An infected individual acquires lifelong immunity against the infecting viral serotype (WHO, 2009) however, secondary infections by another serotype can result in severe pathologies known as DHF and dengue shock syndrome (DSS), which can be lethal (Halstead, 2008).

Mosquitoes will become infected after feeding on human blood infected with the dengue virus during a period of 4 to 5 days from the day the symptoms of dengue fever could be observed. The virus will multiply in the mosquito and after 8 to 14 days, the mosquito would be able to transmit the virus to susceptible humans. Once infected, the mosquitoes remain infective for life (Goma, 1966).

Although dengue viruses are related, the acquired antibodies after being infected with one dengue virus type is unable to prevent a person from being

infected with other types of dengue virus (WHO, 2009). There is no vaccine for dengue fever and the most effective measures to control dengue fever are to eliminate mosquito larvae breeding habitats of *Ae. aegypti* and *Ae. albopictus* (Lambrechts *et al.*, 2010). This step requires a cooperation and continuous effort from the local community (Nasci and Miller, 1996). Currently vector control is conducted after the reports of dengue cases and once the disease is wide spread in the human population, the fogging activities are often less effective (Ang and Satwant, 2001, Rohani *et al.*, 2011).

The first report of DF in Malaysia was in 1902 in Penang and the emergence of DHF was in 1962 (Ang and Satwant, 2001, Nazni et al., 2009). One of the worst incidences of DF occurred in 1998 with 123.4 cases/100,000 population during the global pandemic (Ang and Satwant, 2001). In 2005, the Ministry of Health Malaysia reported that there was an increase in number of cases of DF and DHF, which is 39,654 cases with 107 deaths in 2005 compared to 33,895 cases with 102 deaths in the previous year. From the total cases in 2005, a total of 37,612 cases were dengue fever and the rest of the 2,042 cases were dengue haemorrhagic fever. In 2010, the number of cases increased drastically to 46,171 cases and an increase of 54% of death compared to 2009 with 87 losses of lives (MOH, 2011).

1.6.2 The vector of yellow fever

There are two types of yellow fever which is urban yellow fever that is spread by *Aedes aegypti* and forest/rural yellow fever that is spread by *Aedes africanus, Aedes taylori, Haemagogus sp*. and Sabethes *sp*. Yellow fever is caused by a virus from the Flavivirus genus in the family Flaviviridae (Nasci and Miller, 1996, Varma, 1989). This disease originated from Africa and is endemic in the tropical rain forests in Africa and in Latin America (Varma, 1989, WHO, 2013b). The World Health Organization (WHO) estimates that approximately 200,000 yellow fever cases occur each year and the majority of cases are in sub-Saharan Africa (WHO, 2013b).

Yellow fever is a zoonosis and is essentially a disease of monkeys in the forest, but under certain conditions it can infect humans (Service,

2012). Research findings have shown that yellow fever virus was maintained in the population of monkeys that inhabit the forest canopy, and mosquitoes that breed in the canopy (Varma, 1989). Humans will be infected when they enter areas where the animals' cycle is endemic and when infected mosquitoes come out of the forest. These mosquitoes would instead transmit the disease to those living in nearby villages. This disease has never been reported to occur in Asia despite having an abundance of the vector mosquito, *Ae. aegypti* and susceptible monkey populations (Varma, 1989).

Symptoms experienced by a person when infected with the virus are fever, headache, joint pain, nausea and vomiting. In severe infections, bleeding may also occur (Goma, 1966). Yellow fever spread more quickly than malaria, and when a person is infected, death can occur immediately (Spielman *et al.*, 2001) Unlike DF and DHF, there is a known vaccine for yellow fever called 17D which has been commercially used since the 1950s (Bae *et al.*, 2008).

1.6.3 The vector of Chikungunya

Chikungunya is caused by a virus from alphavirus genus of the Togaviridae family (WHO, 2008b). It originates from tropical Africa. The main virus reservoirs are monkeys, but other species can also be affected (Lahariya and Pradhan, 2006). In Africa, chikungunya is spread through a sylvatic cycle where the virus largely resides in other primates in between human outbreaks. Chikungunya is spread through bites from Ae. aegypti and Ae. albopictus (Enserink, 2007). However, Ae. albopictus has been reported to be the primary vector of this disease. A study conducted in Gabon detected the presence of chikungunya virus in pools of *Ae. albopictus* collected from the field and the presence of this species are more abundant compared to *Ae. aegypti* (Pagès F *et al.*, 2009).

Symptoms of this disease are similar to dengue fever and can be misdiagnosed especially in areas where dengue is endemic. A person suffering from chikungunya could have a high fever up to 40°C, arthritis

affecting multiple joints, headache, conjunctival infection, and slight photophobia (Chhabra *et al.*, 2008).

In Malaysia, chikungunya was first reported in Selangor between 1998 to 1999 and re-emerged in Perak in 2006. Recently there have been an increasing number of chikungunya outbreaks. Rozilawati *et al.* (2011) stated that chikungunya was spread by *Ae. albopictus* in the 2008 outbreak in Johor Bharu. In 2005, an outbreak was reported in the French island of Reunion which was spread by *Ae. albopictus* (WHO, 2008b). In 2009 there were an exceptionally high number of cases reported in India and Southeast Asia especially Thailand (Racaniello, 2009).

1.7 Vector control

The basic principles of a control program are to reduce the breeding sites, reduce the survival rate of the mosquito and/or to prevent man and vector contact (Jayawardene *et al.*, 2011). Mosquitoes cannot be controlled easily or effectively without a deep understanding of their biology. It is important to know where the breeding sites of the mosquitoes are, the biting and resting behaviour of mosquitoes and the flight distance. Mosquito control measures can be directed to control the immature or adult mosquitoes (Goma, 1966, Becker *et al.*, 2003).

There are several ways of controlling a vector population which is through source reduction, biological control, genetic manipulation and chemical control. Traditional control measures that focuses on reducing the population of mosquitoes usually takes a long period of time to become effective (Walker *et al.*, 2011). Mosquito vector control through the usage of chemicals such as insecticides is an effective control measure because of their fast action, but it is toxic to nature (Becker, 2003). The best vector control strategy is to combine source reduction method to eliminate mosquito breeding sites, to educate the general public, law enforcement and the use of a controlled insecticide usage (Lai *et al.*, 2001).

1.7.1 Environmental management and community cooperation

Reducing the population density of mosquitoes by reducing or eliminating mosquito breeding sites or by making water conditions unsuitable for mosquito breeding (Goma, 1966, Ranson *et al.*, 2008) can be effective if sustained.

A sustainable environmental management of the *Aedes* breeding sites includes the participation of health authorities with the education, public service and environmental sanitation sector to build community participation. Manual removal of potential breeding sites of *Ae. aegypti* by communal health workers, paid health-collaborators, school teachers and pupils played a major role in controlling this vector mosquito in Vietnam (Nam *et al.,* 1998, Kay and Nam, 2005, Hales and van Panhuis, 2005). Without the support of the government and the local community, the control strategy such as that would not be as effective (Nam *et al.*, 1998). Numerous structural and community interventions have been effective in interrupting the life cycle of *Aedes* mosquitoes (Jayawardene *et al.*, 2011). Vector-borne disease control program that involves the participation and acceptance of the community, well managed and sustainable is the cause of the program to become successful (Horstick *et al*., 2010). A pilot test conducted in Sri Lanka involving educating school children on the importance of eliminating potential breeding sites of *Aedes* and implementing it in their residential areas has significantly reduce the larval indexes (Jayawardene *et al.*, 2011). A successful vector control program needs to include a cost-effective and well-managed integrated vector management as well as a monitoring and evaluation plan that has to be performed regularly (Horstick *et al*., 2010).

In order to reduce the number of dengue cases, the Vector Borne Disease Control Program in Malaysia was first introduced by the Ministry of Health in 1983. It was aimed to control 7 vector borne diseases: Malaria, Dengue, Filariasis, Japanese Encephalitis, Plague, Scrub Typhus and Yellow Fever (MOH, 2008). To prevent the spread of dengue, in addition to insecticidal treatment, potential breeding site reduction was carried out by trained personnel and ‘10 minutes a day campaign’ aimed to urge members of the

public to set aside 10 minutes a week to clean their houses and dispose of any possible mosquito breeding containers (MOH, 2008). Unfortunately, this campaign was not as successful as the government had hoped due to the lack of community participation and dengue cases continued to increase throughout Malaysia.

1.7.2 Biological control

Biological control is defined as the utilisation of natural enemies to reduce the damaged caused by noxious organism to tolerable level (Debach, 1974). The use of natural enemies or predators such as fish and larvae of *Toxorhynchites* in reducing *Aedes* larvae is non-toxic to the environment. The negative side of this step is that it may take several days to be effective and its implementation measure is quite difficult (Service, 2012). Kweka *et al.* (2011) has reported that aquatic predators of *Anopheles gambiae* larvae have proved to be efficient in reducing the number of larvae in the western Kenya highlands. The naturally existing predators have been reported to coexist in the aquatic habitat with the mosquito larvae and the most efficient predator is the mosquito fish, *Gambusia affins* (Kweka *et al.*, 2011). In Vietnam, the use of *Mesocyclops* copepods which is a predator of first instar larvae has become an effective method of eradicating *Ae. aegypti* mosquitoes (Nam *et al.*, 1998, Kay and Nam, 2005). The introduction of the copepods into the water storage containers and the elimination of other *Aedes* breeding containers with the help of the community through an intensive recycling program has reduced the population size significantly and gradually eliminating the mosquito species in that area (Nam *et al.*, 1998, Kay and Nam, 2005). The use of copepods has also been reported to eradicate local *Ae. albopictus* population in New Orleans by introducing them into tires which are also the mosquitoes' breeding site (Marten, 1990, Nam *et al.*, 1998).

Bacillus thuringiensis var. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) are bacteria with mosquitocidal properties. These *Bacillus* species produce crystalline proteinaceous toxins during sporulation which breakdown the midgut lining when ingested by mosquito larvae and eventually causing death

(Becker, 2003, Zahiri and Mulla, 2006). A study conducted by Lee *et al.* (2008) in Malaysia showed that there was a significant reduction in larval density after 4 weeks of *Bti* treatment. A study also showed that *Bti* did not influence the oviposition behaviour of both field and laboratory strain of *Ae. albopictus in* Clemson, USA (Stoops, 2005). The use of *Bti* and *Bs* are a good form of biological agents due to the ease of being mass produced, highly efficient and environmentally safe (Becker, 2003).

Another example of a biological control method is the use of *Metarhizium anisopliae* fungus to control *Ae. aegypti*. The introduction of *M. anisopliae*-infected males could successfully infect females during the copulation process or through contact and the infection could reduce fecundity and increase mortality of the mosquitoes (Reyes-Villanueva *et al.*, 2011).

1.7.3 Genetic manipulation

Recently, two natural Australian population of *Ae. aegypti* have been successfully infected with *Wolbachia pipientis,* an intracellular bacterium. *Wolbachia* infects the host population through cytoplasmic incompatibility and due to its maternal inheritance, it is rapidly spread throughout the population (Hoffmann *et al.*, 2011). Cytoplasmic incompatibility causes the generation of unviable embryos when an uninfected female mates with an infected male. In contrast, the infected females will produce progeny when they mate with males but their offspring will be infected with *Wolbachia* (Walker et al., 2011, Hoffmann et al., 2011). *Wolbachia* infection aids in reducing *Ae. aegypti* ability to transmit dengue by reducing the ability of the virus to multiply and shortening the mosquitoes' life span (Walker et al., 2011, Hoffmann et al., 2011). A large scale open field trial was conducted in Queensland, Australia (Iturbe-Ormaetxe *et al.*, 2011). Preliminary data showed promising results; after 3 months of weekly releases of 6,000 infected male *Ae. aegypti*, 20% of the population was already infected with the *Wolbachia* bacterium (Coffey, 2011, Iturbe-Ormaetxe et al., 2011).

Another method of vector control by manipulating the genetics of an organism is through the Sterile Insect Technique (SIT). SIT involves releasing sterile insects over an area to mate with the wild insects present. Mating of released sterile males with wild females leads to a decrease in the females' reproductive potential because their offspring are not viable (Klassen and Curtis, 2005). SIT is species-specific and has no effect on other 'non-target' species. SIT was first introduced to control agricultural pests. One of the most successful control measures using SIT was for controlling the *Cochliomyia homonivorax*, the New world Screw Worm in the United States in the 1950s (Klassen and Curtis, 2005). SIT has not been in operational use against mosquitoes due to the damaging effect of radiation to the male mosquitoes (Benedict and Robinson, 2003, Alphey et al., 2010). However, there are researches in Italy that are investigating the potential use of SIT using irradiated *Ae. albopictus* mosquitoes (Vreysen *et al.*, 2007).

Oxitec, a biotechnology company has generated a technology where an insect carries a dominant lethal gene, which is an advancement to the SIT. The release of mosquitoes carrying a dominant lethal (RIDL) genetic system replaces the need for damaging irradiation used in conventional SIT by genetic modification (Alphey *et al.*, 2010). The inclusion of a specific construct causes the overproduction of a specific protein when expressed at a high level is lethal against immature insect (Alphey and Andreasen, 2002) (Alphey et al., 2010 , Harris et al., 2011). RIDL strain of *Ae. aegypti* known as OX513A has been engineered and used in a study that was conducted in Cayman Islands. The 2009 study showed that the RIDL male mosquitoes were successful in competing against the wild type males and suppressing the field population (Harris et al., 2010, Harris et al., 2011, Harris et al., 2012). Other open release of the modified mosquitoes was conducted in Pahang (Malaysia) and Brazil (Oxitec, 2010).

1.7.4 Chemical control

Chemical control by using insecticides is the most popular method for the control of household pests and for public health (Yap *et al.,* 1984, Hemingway and Ranson, 2000). The usage of insecticide treated materials such as curtains, nets and water storage jar covers has been shown to effectively control *Ae. aegypti* mosquitoes (Kroeger *et al.*, 2006).

Ultra Low Volume (ULV) ground aerosol application of insecticides are efficient against adult mosquitoes and the degree of effectiveness is dependent on the dose of the active ingredient in the insecticide used (Mount, 1998). The usage of this outdoor space spraying is an effective method in endemic situations. Thermal fogging and ULV sprays applied by ground vehicle or aerially applied using malathion and pyrethroids have been proven effective in some countries (Lim and Visvalingam, 1990, Perich et al., 2003). In Costa Rica the application of Lambda-cyhalothrin insecticide using the hand held ULV or thermal fogging spray through the front door of houses has proved to be effective in controlling *Ae. aegypti* populations within homes where it is normally hard for insecticides to penetrate using traditional methods (Perich *et al.*, 2003). The use of temephos as larvicides to control *Ae. aegypti* larvae is also a successful method and has been practiced in Thailand (Kay and Nam, 2005).

Another effective method of chemical control is the usage of combined larvicidal-adulticidal ULV formulation. In Argentina, a study has shown that the usage of permethrin as an adulticide and pyriproxyfen an insect growth regulator (IGR) targeted to kill larvae has proved to be effective in field trials against dengue vectors (Lucia *et al.*, 2009).

Another chemical that is used as a vector control tool is the juvenile hormone analogue (JHA). This hormone targets the aquatic larvae of mosquitoes inhibiting metamorphosis of the juvenile stage. A study conducted by Devine *et al.* (2009) observed that pyriproxyfen a JHA which was auto-disseminated by adult *Ae. aegypti* to oviposition sites reduced the number of emerging adult mosquitoes.

Insecticide residual spray (IRS) is another method of chemical control which involves coating the walls and other surfaces of a house with a residual insecticide. The insecticide will kill mosquitoes and other insects that come in contact with these surfaces. IRS does not directly prevent people from being bitten by mosquitoes. Rather, it usually kills mosquitoes after they have fed, if they come to rest on the sprayed surface. This method is more commonly targeted on malaria vectors (Hemingway *et al.*, 2013) and is never practiced on *Aedes* mosquitoes.

Insecticide-treated nets (ITNs) are another method which is more practiced for the control of malaria. In a cluster-randomized trial with permethrin impregnated bednets in Haiti, which was directed towards the control of *Ae. aegypti*, it was observed that the abundance of this mosquito species reduced in homes using the bednets with Breteau Index reduced by 88% (Lenhart *et al.*, 2008).

Although these methods have been proven to reduce the number of dengue vectors, the cases of dengue still persist around the world due to constant human movement that enables the spread of dengue (Horstick et al., 2010). Also, the sizes of cities are expanding and not what is used to be causing control efforts to become harder. Despite these reasons, chemical control can help in reducing the number of dengue cases when the usage is well planed and continued monitoring of the dengue vector is conducted (Horstick et al., 2010) (Pilger et al., 2009).

1.8 Insecticide classification

There are four main groups of insecticide used in public health: pyrethroid, chlorinated hydrocarbons (organochlorine), organophosphate, carbamate and (Lee and Yap, 2003).

1.8.1 Pyrethroids

Pyrethrum is a botanical insecticide, which is extracted from the plant of the Chrysanthemum genus (McLaughlin, 1973). Although pyrethrum is toxic to mammals when ingested, the toxicity when exposed to the skin is low. This is different for arthropods, pyrethrum is very toxic to arthropods even when only exposed to the surface of the cuticle (contact poisons). Pyrethrum is a fast acting insecticide, but the disadvantages are that it is easily degraded when exposed to sunlight and air and the compound is unstable (Stenersen, 2004). The extraction process is expensive and time-consuming. Therefore, scientists have modified the chemical structure of this insecticide and have synthesized analogues (pyrethroid) which is more effective (Lee and Yap, 2003).

Basically, pyrethroid can be divided into four generations. The first generation was in the market in 1949 when allethrin was synthesized based on 22 chemical reactions. It is effective against house flies and mosquitoes, but not effective against cockroaches and other insects (Lee and Yap, 2003, Stenersen, 2004).

The second generation that was introduced in 1965 is tetramethrin. Its knockdown effect is better against flying insects compared with allethrin. Moreover, it can be used together with other compounds. Other insecticide in the second generation is resmethrin, bioresmethrin, d-trans allethrin and phenothrin (Lee and Yap, 2003).

The third generation of pyrethroid appeared in 1972. These include permethrin and fenvalerate, which is the first effective agricultural pyrethroid for its unique insecticidal properties which is photostable and has a residual feature (Lee and Yap, 2003).

Pyrethroid from the fourth generation is 10 times more effective compared to the third generation pyrethroid. The fourth generation insecticides are cypermethrin, bifenthrin, deltamethrin and esfenvalerate. Besides being photostable, they are also stable in the presence of ultra-violet (UV) rays (Lee and Yap, 2003, Miller and Adams, 1982).

Pyrethroids can be further divided into two groups which are type I and type II. The effects of type I pyrethroid (permethrin) are hyperactivity and trembling (seizures) for type II pyrethroid (deltamethrin) the effect is paralysis of the insects. Pyrethroid's overall characteristics as an insecticide are very good and could be the most ideal insecticide, if resistance does not occur (Miller and Adams, 1982).

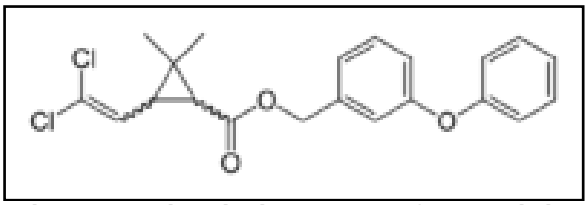


Figure 1.5 Chemical structure of permethrin
(Source: http://en.wikipedia.org/wiki/Permethrin)

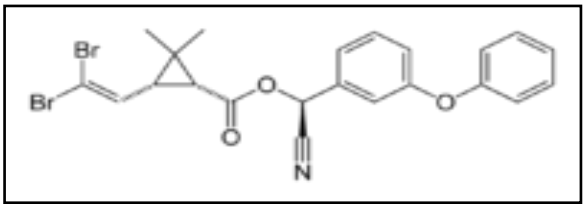


Figure 1.6 Chemical structure of deltamethrin
(Source: http://en.wikipedia.org/wiki/Deltamethrin)

1.8.1.1 Pyrethroid mode of action

Pyrethroids are neurotoxic to insects. Insects that have been exposed to pyrethroid will be agitated, hyperactive, uncoordinated and paralysed. For the flying insects, the knockdown effect is very fast. The symptoms will vary depending on the type and dose of pyrethroid used (Becker, 2010).

Pyrethroids act on the nerve membrane and affect the sodium channels by nerve excitation that occurs as a result of changes in the nerve membrane permeabilities to sodium and potassium ions. Neuro-physiological changes that occur as a result of this action are repetitive firing and the prevention of neuro-muscular transmission (Stenersen, 2004).

Pyrethroids and DDT have similar modes of action. Pyrethroid is chosen to impregnate materials such as bed nets and curtains and also IRS notably for

malaria because it is highly effective and its repellent effect is very strong against the mosquitoes (Chandre *et al.*, 1999). For the control of dengue vector, pyrethroids are normally used in thermal fogging or ultra-low volume sprays. However, pyrethroids are not recommended for the use for larviciding since there are concerns of development of vector resistance towards synthetic pyrethroids and are very toxic to aquatic animals (WHO, 2005b). Pyrethroids have not been shown to have adverse effects on humans when exposed to the public health levels (WHO, 2005b)

1.8.2 Chlorinated hydrocarbon (Organochlorines)

This group of insecticide could be divided into DDT and related compounds such as dieldrin, cyclodienes and hexachlorobenzene (HCB). The chlorine carbon bonds are very strong and do not break down easily. They are very hydrophobic and are chemically unreactive, making them very stable in the environment (Lee and Yap, 2003). Organochlorines are very hazardous to the environment and human health (CDC, 1988)

DDT was the first insecticide that was widely used for the control of agricultural pests and was discovered in 1939 by Paul Hermann Müller (Lee and Yap, 2003, Smith, 1999). It was widely used since it has a long residual effect and is highly toxic to insects (Smith, 1999). DDT affects the sodium channel in the insects' nervous system and acts at the target site by prolonging the inward sodium current and inhibits the increase in potassium permeability (Hassall, 1982). This causes the insect to develop tremors and lead to paralysis and death.

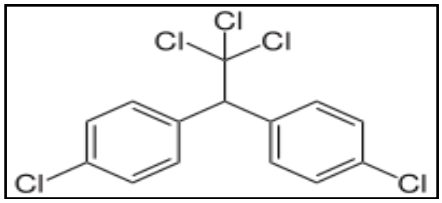


Figure 1.7 Chemical structure of DDT
(Source: http://en.wikipedia.org/wiki/DDT)

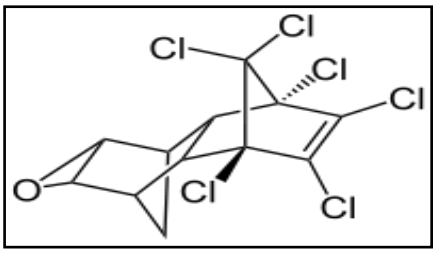


Figure 1.8 Chemical structure of dieldrin
(Source: http://en.wikipedia.org/wiki/Dieldrin)

1.8.3 Organophosphates

The insecticides in this group contain phosphate molecules. They were first discovered by the Germans in the Second World War as a weapon of human warfare (Lee and Yap, 2003). They are chemically unstable and non-persistent in the environment (WHO, 2006a).

Organophosphate insecticides kill insects by binding to and inhibiting acetylcholinesterase (AChE) at synaptic junction of the insect nervous system. This overstimulation of the insects' central nervous system results in death (Becker, 2010). An example of members from the organophosphate group that is used in public health is malathion and fenitrothion for IRs or ULV while temephos is used as a larvicide.

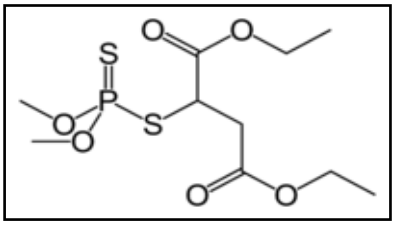


Figure 1.9 Chemical structure of malathion
(http://en.wikipedia.org/wiki/Malathion)

1.8.4 Carbamates

This group of insecticide is identified by the presence of the carbamic acid group. The first successful carbamate, carbaryl was introduced in 1956 (Lee and Yap, 2003). It has relatively low mammalian toxicity and is a broad spectrum insecticide (WHO, 2006b). Due to these characteristics, they have

been widely used in public health and household sectors especially propoxur and bendiocarb.

Carbamates mode of action is the same as organophosphates where they attack the nervous system by binding to AChE and inhibit its function. This causes accumulation of ACh at the available receptors and produces repetitive impulses leading to paralysis due to energy exhaustion (Lee and Yap, 2003).

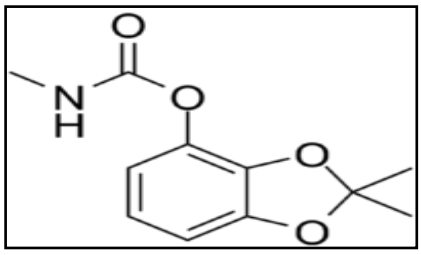


Figure 1.10 Chemical structure of bendiocarb
(http://en.wikipedia.org/wiki/Bendiocarb)

1.9 Insecticide resistance in mosquitoes

More than 500 species of insects are reported to have resistance to one or more groups of insecticide (Nazni et al., 2004, Georghiou, 1990). Of these, 100 species of mosquitoes have been reported to be resistant to one or more of the insecticide and more than 50 species are from the *Culicinae* genus (Nazni et al., 2004, W.H.O, 1992).

According to World Health Organisation (WHO), resistance to insecticides is "the ability that is developed in a strain of an insect species to become tolerant to the doses of a toxic substance that has been proved to be fatal to the majority of normal individuals in the population of the species" (WHO, 2013a). Resistant mosquitoes are rare in the normal population, but the widespread use of insecticide could reduce the number of normal susceptible individuals through the selection pressure it exerts and increase resistant individuals in a population and consequently leading the resistant individuals to eventually become dominant in the population (Hemingway and Ranson, 2000, Becker, 2003).

Cross resistance can occur when an insect population is resistant to two or more classes of insecticides that have similar mechanisms conferring resistance for example DDT and pyrethroids (*kdr* resistance). An insect population could also become multi resistant which means they are resistant due to having different resistance mechanism (an individual insect may have both target site and metabolic resistance or two types or more of the target site/metabolic resistance).

Insecticide resistance in *Ae. aegypti* and *Ae. albopictus* has been reported in countries in the Asia Pacific region such as Malaysia, Thailand, Vietnam, Japan, China, Europe and the countries of Latin America (Ping *et al.*, 2001, Ponlawat *et al.*, 2005, Lima *et al.*, 2011a, Saavedra-Rodriguez *et al.*, 2007).

Frequent use of insecticides with the same mode of action can increase the chances of resistance. For example in Brazil, the implementation of vector control through frequent chemical larviciding by municipal teams from house-to-house has increased the resistance level of *Ae. aegypti* larvae to temephos, an organophosphate (Macoris *et al.*, 2007).

One of the most popular or main insecticide used in *Aedes* control program are pyrethroids but resistance to this insecticide has been reported around the world. In Cayman Islands the *Ae. aegypti* population were highly resistant to DDT and pyrethroids (Harris *et al.*, 2010). In Central Africa, a few populations of *Ae. aegypti* and *Ae. albopictus* were reported to be resistant towards deltamethrin (Kamgang *et al.*, 2011). The development of resistance in *Ae. aegypti* and *Ae. albopictus* towards pyrethroid is becoming more worrying because in epidemic conditions, the control programs of this mosquito species depends greatly on the use pyrethroid insecticide (Saavedra-Rodriguez *et al.*, 2008). During DF epidemic, Ultra Low Volume (ULV) spray and thermal sprays of the insecticide of certain organophosphates and pyrethroids are done to control adult mosquitoes (Paeporn *et al.*, 2004, Ranson *et al.*, 2010). Cross resistance between pyrethroid insecticides can occur in vector mosquitoes due to the fact that these insecticides share the same target site mechanism. This has been reported in *Ae. aegypti* mosquitoes in the North of

Thailand which developed cross-resistance to deltamethrin, permethrin and etofanprox (pyrethroid) (Sathantriphop *et al.*, 2006).

In Makkah City, Saudi Arabia, the frequent use of insecticide in agricultural pest control as well as mosquito control programs has caused for selection of resistance in the natural population of *Ae. aegypti (Al Thbiani et al., 2011)*. This is also true in the case for Malaysian population of *Culex quinquefasciatus*, resistance to fenitrothion is mostly caused by chemicals in the agricultural sector since this insecticide is not used in the Malaysian Vector Control Program (Nazni *et al.*, 2005). Prolonged use and excessive usage of the insecticide may lead to vector resistance and control measures such as thermal spraying would become ineffective (Sathantriphop *et al.*, 2006, Nazni *et al.*, 2005). The usage of agricultural insecticides impacts *Aedes* mosquito control since these vectors could breed in natural pools of water near vegetation such as tree holes (Lee, 2000)

Researchers in Makkah City has concluded that the major cause of resistance in adult *Ae. aegypti* to a few pyrethroid insecticides are due to the frequency of application, mode of action of the insecticides, dispersal of the target mosquitoes and also selling of misbranded insecticides by entrepreneurs (Al Thbiani *et al.*, 2011).

1.9.1 Insecticide resistance of *Aedes* mosquitoes in Malaysia

Malaysia is a developing country and the rapid modernisation has caused the increase in dengue vector breeding sites. This increases the number of dengue cases leading to the over reliance on ‘emergency’ controls rather than constant monitoring and evaluation (Rohani *et al.*, 2011). These emergency controls are normally ULV spraying of chemical insecticides and they are becoming ineffective due to the presence of insecticide resistance in the dengue vectors (Chen et al., 2005b).

Most of the studies conducted are normally focused in Kuala Lumpur and Selangor which are urban cities with high number of dengue cases. Unfortunately, the other states are also having great trouble in controlling

dengue cases and control efforts are being affected due to the development of resistance. There are no reported data of resistance status on a countrywide distribution scale and the resistance mechanisms are less explored by the researchers in Malaysia. The only method in understanding the mechanism of resistance is through the biochemical microassays which only gives an idea of the resistance and not the true mechanisms involved (Chen *et al.*, 2008a, Wan-Norafikah *et al.*, 2008). This is due to Malaysia not having the expertise as well as the proper equipment to conduct molecular studies to identify resistance.

Insecticide susceptibility studies of *Ae. aegypti* and *Ae. albopictus* have been carried out in certain locations. *Aedes aegypti* strains from Selangor (located in central Malaysia) showed resistance towards temephos with an LT_{50} of 36.07 – 75.69 minutes and biochemical assays conducted showed that esterases played a role in this resistance (Chen *et al.,* 2008a). Another study conducted by Hidayati *et al.* (2011) reported that *Ae. aegypti* from Kuala Lumpur were highly resistant to DDT and fenitrothion, moderately resistant to propoxur, tolerant to permethrin and λ-cyhalothrin, and very low resistance to cyfluthrin.

Wan-Norafikah *et al.* (2013) showed permethrin resistance in a Kuala Lumpur strain with a resistance ratio (RR) of 2.15 when compared to laboratory susceptible strain. They also concluded that the resistance was due to oxidases after conducting mixed function oxidases (MFOs) microassay and not *kdr* alleles (Wan-Norafikah *et al.*, 2008). Multiple resistance to both permethrin and DDT was observed in this species from Kuala Lumpur and strains from Kelantan and Johor showed moderate resistance towards permethrin in a study conducted by Rohani *et al.* (2001). *Aedes albopictus* from Penang was resistant towards deltamethrin and permethrin which showed a RR of 8.99 and 7.71 for each of the insecticide respectively after using the topical application method (Chan *et al.*, 2011).

Different species of mosquitoes from the same area could have different resistance status towards the same insecticide. In a study area in Kuala Lumpur, *Ae. aegypti* were more resistant than *Ae. albopictus* towards

temephos (Chen et al., 2005b, Wan-Norafikah et al., 2010). This could be because *Ae. aegypti* prefers to rest indoors and they are more exposed to household insecticides compared to *Ae. albopictus* which prefers to rest outdoors (Chen et al., 2005b).

Another important point is that different life stages of mosquitoes also differ in their resistance profiles and the detoxification enzymes involved during the immature stage would not necessarily be over expressed in the adult stage. This could be shown in the study by Selvi *et al.* (2010) in Malaysian population of *Ae. albopictus* where adult mosquitoes showed a higher expression of esterases compared to the larval stage.

1.9.2 Mechanism of insecticide resistance in mosquitoes

The two best studied insecticide resistance mechanisms are alterations in the target site of the insecticide which leads to changes in the sensitivity of the target site and alterations in activities of enzyme families that causes an increase in the detoxification process of the insecticide (metabolic resistance) (Bonnet *et al.*, 2009, Ranson *et al.*, 2010, Saavedra-Rodriguez *et al.*, 2008). Behavioural resistance and reduced cuticular penetration are observed in vector mosquitoes but are less explored. The two important resistance mechanisms and their relative role in conferring resistance to each insecticide class are shown in Table 1.1.

Insecticide	Mechanism of resistance				
	Metabolic resistance			Target-site resistance	
	Esterases	Monooxygenases (P450s)	Glutathione-S-Transferases	Kdr	Ace1
Pyrethroids	*	**	**	**	
DDT		*	*	**	
Carbamates	*	*			**
Organophosphates	**	*	*		**

Table 1.1 Major mechanisms conferring resistance to important classes of insecticides in mosquitoes. Adapted from (Nauen, 2007).
* indicates this mechanism has been described but is considered to be of less importance;
** indicates an important resistant mechanism.

1.9.2.1 Behavioural resistance

Behavioural resistance occurs when insects are able to avoid contact with insecticides by an inherited change in behaviour. (Marquardt and Kondratieff, 2005). These behavioural changes could be stimulus dependent or stimulus independent. Stimulus dependent response is when the mosquitoes sense the presence of insecticide and totally avoid the insecticide treated surfaces. Stimulus independent response is when a mosquito population starts occupying an area that is not treated with insecticide (Collier-mosquito.org, 2014).

Insecticides such as DDT and permethrin also influence behavioural changes in the insects, by reducing the rate of mosquito entry into houses, increasing the rate of early exit from houses and inducing a shift in biting times (Miller *et al.*, 1991).

The reason behind the behavioural changes which could either be due to a response to irritant insecticides or a genetic trait are less explored (Pates and Curtis, 2005). Behavioural resistance is not easy to assess hence the affect of this mechanism is not well understood.

1.9.2.2 Reduced cuticular penetration resistance

This mechanism of insecticide resistance is caused by the thickening or changes in the chemical composition of the insect cuticle. An insecticide would only be effective when it could reach the target site and since the insecticide could not penetrate the cuticle, the insecticide would be ineffective (Lee and Yap, 2003). This resistance mechanism will delay the rate of insecticide penetration into the insect body and cause the detoxification mechanisms to take effect.

A study conducted by Djouaka *et al.* (2008) identified an over expression of two cuticular precursor genes belonging to the low-sequence complexity group CPLC in two population of *An. gambiae* from Southern Benin and Nigeria. In Kwazulu, South Africa cuticular thickening of the *An. funestus* was

associated with pyrethroid resistance by measuring the cuticular thickness and the susceptibility towards the insecticide (Wood *et al*., 2010, 2008).

Insects with this type of resistance mechanism normally would not have a resistance level more than 3-fold compared to susceptible insects. However, if this mechanism co-existed with other resistance mechanism it will increase the level of insecticide resistance significantly (Lee and Yap, 2003) as seen for *An. gambiae* in Benin (Djouaka *et al.*, 2008) where over expression of P450s and cuticular genes combined to confer a high resistance level.

1.9.2.3 Target site insensitivity

Target site resistance occurs through an amino acid substitution in the target site receptor that reduces the binding to the insecticides. This resistance could occur in the voltage gated sodium channel, acetylcholinesterase (Ache) and GABA receptors (Hemingway and Ranson, 2000, Sathantriphop *et al*., 2006).

1.9.2.3.1 Knockdown resistance (*kdr*)

Mutations or a replacement at a single codon in the voltage gated sodium channel cause knockdown resistance (*kdr*) which is a reduction in the nerve sensitivity to pyrethroids and DDT (Soderlund, 2008). Some of these mutations have been related with a reduction of electrophysiological sensitivity and less efficient functionality (Lima *et al.*, 2011b).

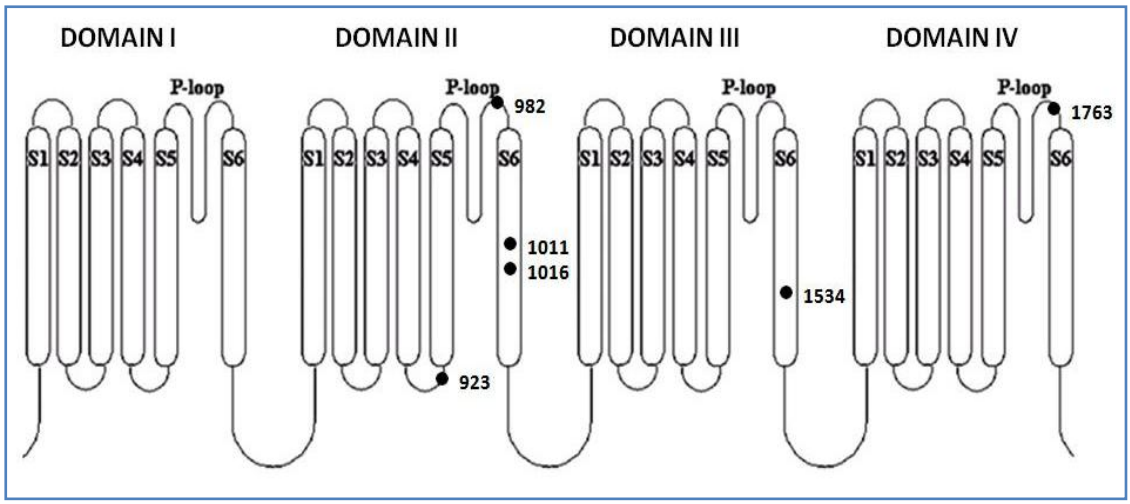


Figure 1.11 Schematic of the transmembrane voltage-gated sodium channel (VGSC). Figure from (O'Reilly *et al.*, 2006). The location of mutation is represented by black dots and are numbered according to the sequence of the housefly VGSC.

The voltage gated sodium channel is the target site for DDT and pyrethroids. The channel contains four homologous domains and each of the domains consists of six hydrophobic subunits (S1 – S6) (Usherwood *et al.*, 2005). Amino acid substitutions at five residues in the voltage gated sodium channel have been associated with knockdown resistance in *Ae. aegypti* (Figure 1.11). *Kdr* mutations such as I1011M, I1011V, V1016G and V1016I have been found in knockdown resistant population of *Ae. aegypti* in Vietnam and also Latin America (Saavedra-Rodriguez *et al.*, 2008, Kawada *et al.,* 2009). A study conducted in Martinique revealed high frequencies of V1016I *kdr* mutation in the *Ae. aegypti* population and this mutation conferred resistance against deltamethrin insecticide (Marcombe *et al.*, 2009). In *Ae. albopictus* , the first putative F1534C *kdr* mutation was reported in Singapore (Kasai *et al.*, 2011). However, until now no *kdr* mutation was identified at the molecular level in Malaysia. More detailed explanation of the *kdr* mutation in *Ae. aegypti* is in Chapter 4.

Overall, knockdown resistance results in a broad resistance towards pyrethroid, DDT and DDT analogues. Knockdown resistance towards pyrethroid and DDT was first detected in 1951 in house flies (Becker, 2010, Soderlund, 2008). A comparison of *Musca domestica* sodium channel DNA sequence identified a single-point mutation having *kdr* phenotype and the

substitution of amino acids Leucine to Phenylalanine (L1014F) (Williamson et al., 1996, Soderlund, 2008). This mutation was also discovered in other arthropod species such as in Culex *pipiens* (Martinez-Torres *et al.*, 1998), *Cx. quinquefasciatus* in Sri Lanka (Wondji et al., 2008b) and *An. gambiae* and has become one of the most common *kdr* mutation (Ranson *et al*., 2000, Kawada *et al*., 2009, Soderlund, 2008). In *An. gambiae* there are two types of *kdr* allele which is *kdr* L1014F (Mainly in West Africa) and *Kdr* L1014S (mainly located in East Africa). A new mutation was recently discovered in *An. gambiae* s.s. which occurs on only a single long-range haplotype also containing *kdr* 1014F mutation. The new mutation N1575Y and 1014F haplotype was found in both M and S molecular forms of *An. gambiae* in West/central Africa (Jones *et al.*, 2012).

1.9.2.3.2 Insensitivity of Acetylcholinesterase (Ache)

Ache is important for hydrolysis of acetylcholine at the cholinergic nerve synapses. It initiates action potential at the postsynaptic receptor. When organophosphates and carbamates bind to acetylcholine esterase, it inhibits Ache and prolongs neuroexcitation leading to the death of the mosquito (Lee and Yap, 2003, Marcombe et al., 2009). In *Cx. pipiens*, a single mutation on the coding sequence of *Ace-1* gene is responsible for the substitution of a glycine to a serine at the position 119 (G119S) (Weill *et al.*, 2003). This mutation has a negative impact on mosquito fitness by affecting the enzyme function (Bass and Field, 2011). Insensitivity to Ache has been reported in *Cx. pipiens* (Hemingway, 1992, Weill et al., 2003), *An. gambiae* (Weill *et al.*, 2003) and *Blatella germanica* (Hemingway *et al.*, 1993). Currently, there is no known mutation associated with target site insensitivity in *Ace-1* in *Ae. aegypti* (Vontas *et al.*, 2012).

1.9.2.3.3 Insensitivity of GABA receptor

Ɣ-aminobutyric acid (GABA) is the major neurotransmitter for fast inhibitory synaptic transmission in both vertebrates and invertebrates (Olsen and Macdonald, 2002, ffrench-Constant et al., 2004). Binding of GABA to its receptor activates chloride ion selective channel (Hemingway *et al.*, 2004). The ion channels are the target site of cyclodienes (dieldrin), phenylpyrazoles and fipronil. GABA receptors are composed of 5 subunits, which arrange around the central ion channel (Hemingway *et al.*, 2004). Resistance to dieldrin seems to be related to amino acid replacements coded by single point mutations in the GABA receptor subunit gene (termed Resistance to dieldrin gene, *Rdl*) in several insect species (ffrench-Constant *et al.*, 2004).

Genetic mapping of dieldrin resistance in *Drosophila melanogaster* indicated that resistance was conferred by *Rdl* on the left arm of chromosome III at map position 66F (ffrench-Constant and Roush, 1991). A mutation at a single codon in the GABA receptor gene, a substitution of alanine at position 302 to either a serine or a glycine, conferred dieldrin resistance in *Drosophila* (ffrench-Constant *et al.*, 1993). The mutation of alanine to serine or glycine has been observed in resistant strains of mosquitoes and the species to have been analysed so far for this mutation are *Ae. aegypti* (Thompson *et al.*, 1993, Hemingway *et al*., 2004), *An. funestus* (Wondji *et al.*, 2011) *An. gambiae* and *An. arabiensis* (Du *et al.*, 2005). This mutation was also detected in *Cx. quinquefasciatus* and *Ae. albopictus* from La Reunion island (Tantely *et al.*, 2010).

1.9.2.4 Metabolic resistance

Metabolic resistance is caused by alterations in levels or activities of detoxification enzymes; elevated activities of cytochrome P450 monooxygenase, glutathione-s-transferase (GST) and carboxylesterases. These enzymes act to metabolize insecticide to non-toxic materials with a very fast rate, or reverse binding of the insecticide (hijacking process) causing it to no longer become effective (Hemingway et al., 1998,

Nazni et al., 2004). *Aedes aegypti* have more detoxification genes compared to *An. gambiae* or *Drosophila melanogaster* (Strode *et al.*, 2008) as shown in Table 1.2.

	D. melanogaster	***An. gambiae***	***Ae. aegypti***
Esterases	26	40	49
Monooxygenases (P450s)	86	105	160
Glutathione-S-Transferases	37	28	26

Table 1.2 Number of detoxification genes relating to three insect species.
Adapted from (Strode, 2008).

1.9.2.4.1 Cytochrome P450s

Cytochrome P450 monooxygenases are a large and diverse family of hydrophobic, heme-containing enzymes involved in the metabolism of numerous endogenous and exogenous compounds (Hemingway *et al.*, 2004). The action of cytochrome P450s generally results in the detoxification of the substrate. There are many reports demonstrating elevated P450 monooxygenase activities in insecticide-resistant mosquitoes, and causing metabolic resistance to all classes of insecticides (Li *et al.*, 2007).

Cytochrome P450 monooxygenases play a role in pyrethroid resistance in insects. Marcombe *et al.* (2009) reported that P450 detoxification reduced the effectiveness of deltamethrin space sprays operations in Martinique. In *Ae. aegypti* mosquitoes, there are at least 160 full-length, an increase of 52% and 86% compared to *An. gambiae* and *D. melanogaster* respectively (Strode et al., 2008). In *Aedes* the CYP9 family has expanded dramatically and been implicated in pyrethroid resistance (Strode *et al.*, 2008, Marcombe *et al.*, 2009, Stevenson *et al.*, 2012, Bariami *et al.,* 2012). A study regarding temephos resistance in *Ae. aegypti* showed an up-regulation of CYP6 and CYP9 families. Four P450s were over-expressed in the resistant compared to the susceptible strain; CYP6M6, CYP6Z6, CYP9J23 and CYP9J22 (Marcombe *et al.*, 2009).

1.9.2.4.2 Glutathione-s-transferase (GST)

These groups of enzyme conjugates reduced glutathione to the reactive electrophilic centers of xenobiotics which produces formation of a water-soluble and less reactive product. GSTs are also involved in binding and sequestration (Li *et al.,* 2007).

Elevated levels of GST have been associated with resistance to DDT and pyrethroids in *Ae. aegypti* (Strode *et al.*, 2008). Some of these enzymes could catalyze DDT to non-insecticidal product, DDE and others are able to metabolize insecticides or its metabolites. GSTs could be divided into three main groups; cytosolic, microsomal and mitochondrial (Lumjuan *et al.*, 2007). The majority of GSTs are cytosolic and there are six groups of cytosolic GST; Delta, Epsilon, Omega, Sigma, Theta and Zeta (Lumjuan *et al.*, 2005). Delta and Epsilon are classes which are specific to insects and may play a role in insecticide resistance (Lumjuan *et al.*, 2005, Strode *et al.*, 2008). Eight epsilon GST genes have been identified in *Ae. aegypti* and GSTe2, GSTe5 and GSTe7 are over-expressed in mosquitoes that are resistant to DDT and permethrin (Lumjuan *et al.*, 2005).

1.9.2.4.3 Carboxylesterases

Carboxylesterase resistance mechanism is involved in organophosphate and carbamate resistance in insects (Bass and Field, 2011). The mechanisms include gene amplification, up-regulation, and/or coding sequence mutations, resulting in enzymes that can hydrolyses or sequester insecticide molecules with greater efficiency (Li *et al.*, 2007). This is true in the case of *Ae. aegypti* population in Martinique where resistance to temephos is associated with elevated carboxylesterase activity (Marcombe *et al.*, 2009).

1.10 Background of research

There is a crucial need to fill the gap in our knowledge about resistance profiles to the main insecticide classes and also the extent (distribution and level) of resistance across Malaysia since most studies only focus on the capital city. This is to implement suitable control programs to different states. Also, no molecular based studies have been conducted in understanding the insecticide resistance mechanisms in *Aedes* mosquitoes in Malaysia. The need for elucidating resistance mechanism is important to inform the Ministry of Health on the choice of suitable insecticides and improve resistance management strategies in Malaysia. This research aims to fill these gaps, by characterising insecticide resistance in the *Ae. aegypti* and *Ae. albopictus*.

1.11 Research Objective

To elucidate the mechanisms of insecticide resistance in Malaysian field populations of *Ae. aegypti* and *Ae. albopictus* using genetic and molecular approaches.

1.12 Research Aims

- Assess the susceptibility status of *Ae. aegypti* and *Ae. albopictus* in Malaysia against main insecticides.
- Elucidate the mechanisms associated with insecticide resistance in *Ae. aegypti* across Malaysia.
- Investigate the role of target-site resistance and metabolic resistance mechanisms in conferring insecticide resistance in *Ae. albopictus* across Malaysia.

2.0 GENERAL MATERIALS AND METHODS

2.1 Mosquito samples

Field *Ae. aegypti* and *Ae. albopictus* were collected during two months (July and August) in 2010 across Malaysia. Ovitraps was set up in four states in Malaysia; Penang (Northwest), Kota Bharu (Northeast), Kuala Lumpur (Centre) and Johor Bharu (South). The sites were chosen because it encompassed the whole of Malaysia from north to south and they represented the different geographical landscape in Malaysia such as urban areas (Penang, Kuala Lumpur and Johor Bharu) and rural areas with more vegetation (Kota Bharu).

Approximately 80 ovitraps was set up in two collection sites in each state. The collection sites were residential areas that had numerous cases of dengue outbreaks and are regularly sprayed with insecticide mainly permethrin and malathion using thermo fogging by the Health Ministry. The traps were collected after five days to prevent the eggs from hatching and the larvae emerging into adults.

Figure 2.1 A map of Malaysia showing the four states of sample collection

Collection of ovitraps was done by pouring all the water containing larvae into a large and secure plastic bottle. The wooden paddle was kept carefully ensuring that the eggs on the paddle would not be damaged. The traps were also kept to be rinsed out in the lab. The location of the ovitraps was also recorded to make sure none are left behind.

Natural breading site collection was also done. Larvae were collected from old tyres, flower pots, tree holes and containers that held water. The larvae were either picked up by using pipettes or the water was poured into a secure container. All the samples were brought back to the lab to be reared.

2.1.1 Mosquito Rearing in Malaysia

Both *Aedes* species from all four sites were brought to the Vector Control Research Unit (VCRU) in Penang. The water containing larvae were poured into enamel trays and fed with larval food which contains grounded dog biscuit, beef liver, powdered milk and yeast at a ratio of 2:1:1:1. Pupae were collected into plastic pots filled with water and transferred into a cage.

Once adults had emerged, the mosquitoes were picked one by one using a manually operated aspirator. The morphological characteristics, mainly the pattern on the thorax were observed and the two *Aedes* species were identified and put into two separate cages. The mosquitoes were fed with 10% sucrose solution soaked in cotton wool. Once all the adults had emerged and separated, they were given a blood meal. Blood feeding was done by using white lab mice or rats depending on the numbers of mosquitoes in the cage. The VCRU does have the ethical board approval to use the mice or rats in feeding of the mosquitoes. The mouse or rat was put in a small wire mesh cage to ensure it would not move when the mosquitoes were feeding.

Two days after the blood meal, a wet filter paper folded into a shape of a cone in a petri dish was placed into the cage for collection of eggs. The filter paper was then taken out of the cage and the cone was inverted making sure that the eggs are now on the inner surface of the filter paper. It was dried at room temperature to ensure that the embryos would develop well in the eggs and

the hatching rate would be uniformed. After being completely dried, the filter paper was kept in a sealed plastic bag and labelled. The mosquitoes were given a blood meal a few times to obtain sufficient numbers of mosquitoes for the study. Dead adult mosquitoes were also collected and put into a few eppendorf tubes. Holes were made on the side of the eppendorf tubes and placed into an airtight plastic bag containing silica gel to prevent mould from growing on the mosquito carcasses. Both the filter papers and dead mosquitoes were brought back to Liverpool School of Tropical Medicine (LSTM).

2.1.2 Mosquito Rearing In Liverpool

The filter papers containing eggs from both *Ae, aegypti* and *Ae, albopictus* for four Malaysian strains were hatched in the insectary at LSTM. The eggs were hatched in plastic trays filled with water and supplemented with approximately 100ml of hay infusion solution. Once the F_1 generation of eggs had hatched into first instar larvae, they were fed with grounded fish food (Tetra flack fish food) and yeast. Pupae were collected into plastic pots and put into cages. Once adult mosquitoes emerged, they were given 10% sucrose solution soaked in cotton wool and replenished on a daily basis. Five to seven day old mosquitoes were blood fed using the Hemotek membrane blood feeding system and they were blood fed every week to increase the number of eggs produced. Wet filter was put into the cage two days after the blood feeding. The filter paper was dried. The process was repeated using the same method as described above for the rearing the mosquitoes in Malaysia. Adult mosquitoes were maintained in 30cm x 30cm x 30cm cages at a room temperature of 27 ± 2^0C with relative humidity of 70 ± 10%.

2.1.3 Larval Bioassays

The larval Bioassays were conducted according to WHO guidelines (WHO, 2000, WHO, 2005a). 1ml of temephos insecticide (1 g/L of original concentration) (Sigma Aldrich) was diluted with ethanol and mixed with 249ml

distilled water. 4 replicates of 10 different concentrations between 0.002ppm to 0.075ppm and ethanol only control were tested on 25 late third instar to early fourth instart larvae. The mortality was recorded after 24 hours of exposure. Larvae that were unable to swim up to the surface were counted as dead and the larvae that had pupated were omitted from the final total. Lethal concentration that killed 50% of the tested samples was calculated using probit analysis (PASW statistics 18 software). Resistance ratio (RR) was also calculated; the susceptible strain for *Ae. aegypti* was from New Orleans and for *Aedes albopictus* was from Vector Control Research Unit (VCRU).

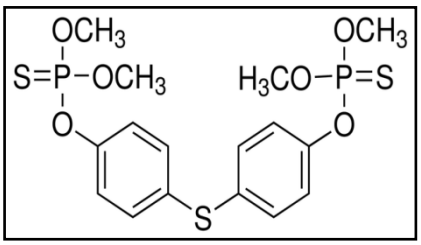


Figure 2.2 Chemical structure of temephos
(Source: http://www.sigmaaldrich.com/catalog/product/supelco/ps665?lang=en&region=GB)

2.1.4 Adult Bioassays

Bioassays were carried out according to WHO approved protocol (WHO, 1981). Bioassays were conducted on F_2 generation of *Ae. aegypti* and *Ae. albopictus* mosquitoes. 2 to 5 days old non-blood fed adult mosquitoes were used. The bioassays were done in a separate room from the insectary where the *Aedes* mosquitoes were reared and with a room temperature of 25 ± 2^0C with 70 ± 10% relative humidity. 25 adult mosquitoes were aspirated from the rearing cage into a holding tube and left in the tube for 1 hour to ensure that all the mosquitoes are still alive and not injured before carrying out the test. The mosquitoes were then blown gently into an exposure tube (with the specific insecticide-impregnated paper) which is placed in a vertical position for one hour. The mosquitoes were then gently blown back into the holding tube and left in a vertical position for 24 hours with a 10% sucrose solution cotton wool placed on top of the tube. The mortality rate was recorded after 24 hours. The bioassays were conducted on 100 female (some were 125) and male mosquitoes, with 4 replicates of 25 mosquitoes per tube. The

insecticides that were tested are: 0.75% Permethrin (Type I pyrethroid), 0.05% Deltamethrin (Type II pyrethroid), 4% DDT (organochlorine), 4% Dieldrin (organochlorine), 0.1% Bendiocarb (Carbamate) and 5% Malathion (organophosphate). The standard error was calculated for all the results obtained.

The effect of pre-exposure to the synergist, piperonyl butoxide (PBO) was also assessed to investigate the potential role of cytochrome P450s in the resistance mechanisms. Adult 2 – 5 days old mosquitoes were exposed to papers impregnated with 4% PBO for one hour and then immediately exposed to four insecticides; permethrin, deltamethrin, DDT or bendiocarb using WHO susceptibility test kits. Mortality was scored after 24 hours.

Survivors after the bioassays were stored in a labelled eppendorf tube and kept in the -80^{0}C freezer for DNA and RNA extraction. Dead mosquitoes were also kept in a labelled eppendorf tube in silica gel for later DNA extraction. These samples are later referred to as alive and dead mosquitoes.

2.2 Genetic Characterisation of Mosquitoes

2.2.1 Genomic DNA extraction

Genomic DNA was extracted using the Livak method (Livak, 1984). Prior to the DNA extraction, Livak grind buffer was prepared using 1.6ml 5M NaCl, 5.48g sucrose, 1.57g Tris, 10.16ml 0.5M EDTA, 2.5ml 20% SDS and sterile water to make up the volume of solution to 100ml. The solution was then filtered sterilize and divided into 5ml aliquots and stored at -20°C until required.

Genomic DNA was first extracted from 50 individual F_0 field mosquitoes for both *Aedes* species from all four strains (Penang, Kota Bharu, Johor Bharu and Kuala Lumpur) to determine the species identity of all the samples. The Livak grind buffer was pre-heated in a water bath at 65°C. A single mosquito was transferred into a 1.5ml eppendorf tube and 50µl of the pre-heated Livak grind buffer was pipetted into the tube. The mosquito was ground and

homogenised with a plastic pestle using a battery operated grinder and 50µl of the Livak grind buffer was used to clean the pestle and added into the tube. The sample was incubated at 65°C for 30 minutes, and then microfuged briefly to collect condensation. 14µl of 8M potassium acetate was added to the sample and mixed before incubation on ice for 30 minutes. The sample was then centrifuged at 13,000rpm for 20 minutes at 4°C. The supernatant was transferred into a new tube and precipitated by adding 200µl of 100% ethanol. The sample was centrifuged again at 13,000rpm for 15 minutes at 4°C. The supernatant was discarded and rinsed with 100µl of ice-cold 70% ethanol. The DNA pellet was left on the bench to dry for approximately 1 hour and then re-suspended with 100µl of distilled water and incubated at 65°C for 10 minutes. The DNA concentration was measured with Nanodrop and stored at -20°C until required.

2.2.2 Species Identification

Molecular identification to the species level was carried out using a Polymerase Chain Reaction (PCR) based method described by Beebe *et al.* (2007) to differentiate between *Ae. aegypti* and *Ae. albopictus*. The primers used for the amplification were, forward primer ITS1A, 5'-CCTTTGTACACACCGCCCGTCG, and reverse primer ITS1B, 5'-ATG TGT CCT GCA GTT CAC A. The amplification of ITS1 (internal transcribed spacer region 1) was carried out on genomic DNA from 50 individual F_0 field mosquitoes from all 8 populations. All PCR reactions were conducted in 0.2ml PCR strip tubes. The final 15µl PCR mixture contained 1µl of genomic DNA (10ng on average), 1.5µl of 10X KAPA Taq buffer A (KAPA Biosystems), 0.12µl of 5 U/µl KAPA taq, 0.12µl of 25µM dNTP, 0.75µl of 25µM $MgCl_2$, 0.51µl of each primer and 10.49µl of dH_2O. The PCR parameters were 95°C for 5 minutes and 35 cycles of 94°C for 30 seconds, 51°C for 40 seconds and 72°C for 45 seconds, followed by a final extension step of 72°C for 10 minutes. The PCR products were size separated on a 1.5% agarose gel stained with ethidium bromide (0.5µg/µl) and visualised using a gel imaging system to confirm the product size. Then, restriction analysis was carried out

on the PCR product. This was done in 0.2ml PCR strip tubes using 5µl of PCR product, 1µl of *RsaI* buffer, 0.2µl of 2 units of *RsaI* enzyme and 3.8µl of dH_20. The mixture was incubated at 37°C for 2 hours and then size separated on a 3.0% agarose gel stained with ethidium bromide (0.5µg/µl) at 100V for 30 minutes. The gel was also visualised using the gel imaging system (Figure 2.3).

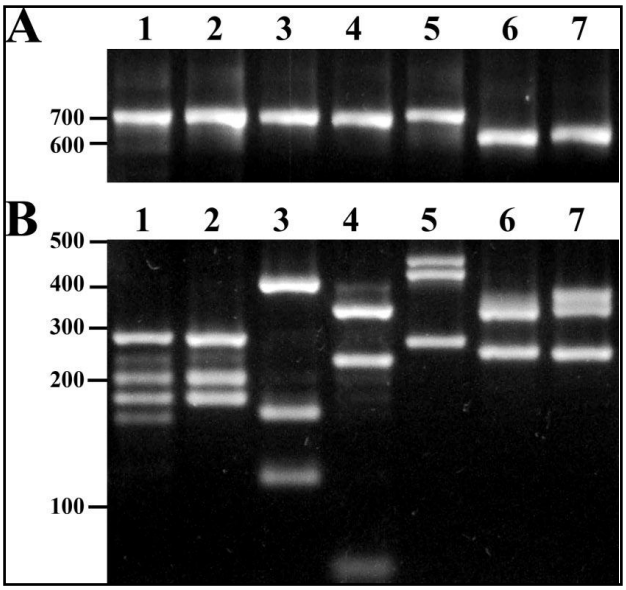


Figure 2.3 PCR product size separated on agarose gel for species identification PCR. (A) PCR amplified ITS1 (B) Digested PCR product with *RsaI* enzyme. Lane 1 & 2: Ae. albopictus, lane 3: Ae. aegypti
Taken from Beebe *et al.* (2007)

2.2.3 Knockdown resistance (*kdr*) frequency genotyping

2.2.3.1 Knockdown resistance in *Aedes aegypti*

The presence of the *kdr* mutation in *Ae. aegypti* was assessed from all four populations using pyrosequencing method. From past literature it was observed that there were three *kdr* mutations in *Ae. aegypti* which were in Exon 20 [I1011V (or M)] (Brengues *et al.*, 2003), Exon 21 [V1016I (or G)] (Saavedra-Rodriguez *et al.*, 2007) and Exon 31 [F1534] (Harris *et al.*, 2010).

30 F_0 *Ae. aegypti* mosquitoes from each population were tested (preliminary test) for the presence of these mutations. Subsequently, the potential role of these *kdr* mutations in the resistance to pyrethroids or DDT was assessed by

establishing the correlation between genotypes and resistant phenotype. 25 dead and 25 alive F_2 mosquitoes from each population that were exposed to permethrin, deltamethrin and DDT were then tested.

The technique used to genotype the *kdr* mutations and to determine the *kdr* frequency is pyrosequencing. Pyrosequencing is a method of DNA sequencing based on the method of 'sequencing by synthesis'. It is a sequence–based detection technology that allows rapid and accurate quantification of variation in certain sequences. This sequencing method was chosen because it was a method that could test a lot of samples at a time and the results obtained were easily obtained and analysed. Test for genotype: phenotype association and test for Hardy-Weinberg equilibrium was done using Chi square test as done by Wondji *et al.* (2008a).

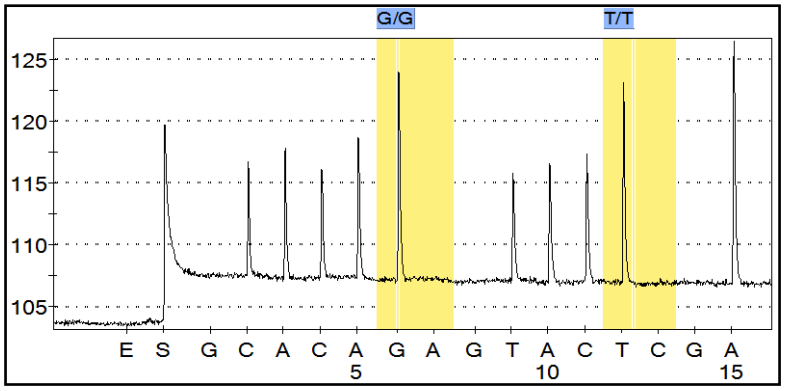


Figure 2.4 Pyrosequencing program traces. An example for *kdr* 1016 mutation.

2.2.3.2 Designing of pyrosequencing PCR primer

The primers were designed using the Primer3 website (http://frodo.wi.mit.edu/primer3/). The sequence for the voltage gated sodium channel gene, AAEL006019 gene in *Ae. aegypti* were located and copied to help in the design of the primers from the vectorbase website (http://www.vectorbase.org/). The nucleotide position of the mutation and the sequence to analyse were both taken into account in designing the primers for pyrosequencing. Three primers were design for each *kdr* mutation; a forward primer, a reverse primer and a sequencing primer. The reverse primer is biotinylated (Table 2.1).

Primer Name	Sequence	Modification	Reference
Ae*kdr*1016pyrF	CTTTCGTGCTAACCGACAAATT	Biotinilated	Wondji (Unpublished)
Ae*kdr*1016pyrR-bio	AAAAGAATCGGAACGAAAACAG G		
Ae*kdr*1016pyrseq	AATTGTTTCCCACTCG		
Sequence to analyse	CACAG A/G G/T ACT C/T AACCT		
Ae*kdr*1011pyrF	TATGCTTGTGGGTGACGTGT	Biotynilated	Wondji (Unpublished)
Ae*kdr*1011pyrR-bio	GCTGCTAGCACGCCTCTAAT		
Ae*kdr*1011pyrseq	TTCTTTTTGGCCACCG		
Sequence to analyse	TAGTG A/G T A/G GGAAATCTAG		
Ae*kdr*1534pyrF	TCGCGGGAGGTAAGTTATTG	Biotynilated	Wondji (Unpublished)
Ae*kdr*1534pyrR-bio	CGATGATGACACCGATGAAC		
Ae*kdr*1534pyrseq	TACTTTGTGTTCTTCA		
Sequence to analyse	TCATCT G/T CGGGTCGT		

Table 2.1 Primers used for pyrosequencing. Highlighted nucleotides show position of mutation.

2.2.3.3 PCR for pyrosequencing

A PCR amplification of the genomic fragment to sequence was first carried out before the pyroquencing assay. Three primer sets were used for the PCR mixture to detect three different *kdr* mutation; *kdr*1011, *kdr*1016 and *kdr*1534. All PCR reactions were conducted in 0.2ml PCR strip tubes using Qiagen HotStar® Taq DNA polymerase reagents. The final 15µl PCR mixture contained 1µl of genomic DNA, 1.5µl of 10X PCR buffer, 0.12µl of HotStar Taq, 0.12µl of 25µM dNTP, 0.9µl of 25µM $MgCl_2$, 0.51µl of forward primer, 0.51µl of biotinylated reverse primer and 10.34µl of dH_2O. The PCR parameters were 95°C for 15 minutes and 50 cycles of 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by an extension step of 72°C for 5 minutes. The PCR products were size separated on a 1.5% agarose gel stained with ethidium bromide (0.5µg/µl) and visualised using a gel imaging system to confirm whether the PCR amplification succeeded.

2.2.3.4 Pyrosequencing assay

The pyrosequencing assay was conducted according to the protocol described previously by Wondji et al (2008a). The biotinylated PCR products were immobilized to streptavidin-coated Sepharose beads. The biotin labelled amplicon strands were separated using the Vacuum Prep worktable and tool. The beads containing the biotin labelled products were released in a PSQ 96 plate prefilled with annealing buffer. The sample plate is then heated on a heating block at 80°C for 2 minutes to anneal the primers. The samples are left to cool at room temperature and continued with the sequencing reaction. The principle of pyrosequencing is shown in Appendix 8.3 (taken from www.qiagen.com).

2.2.4 Microarray

There are a few steps in conducting a microarray experiment. The steps are summarized in Figure 2.5.

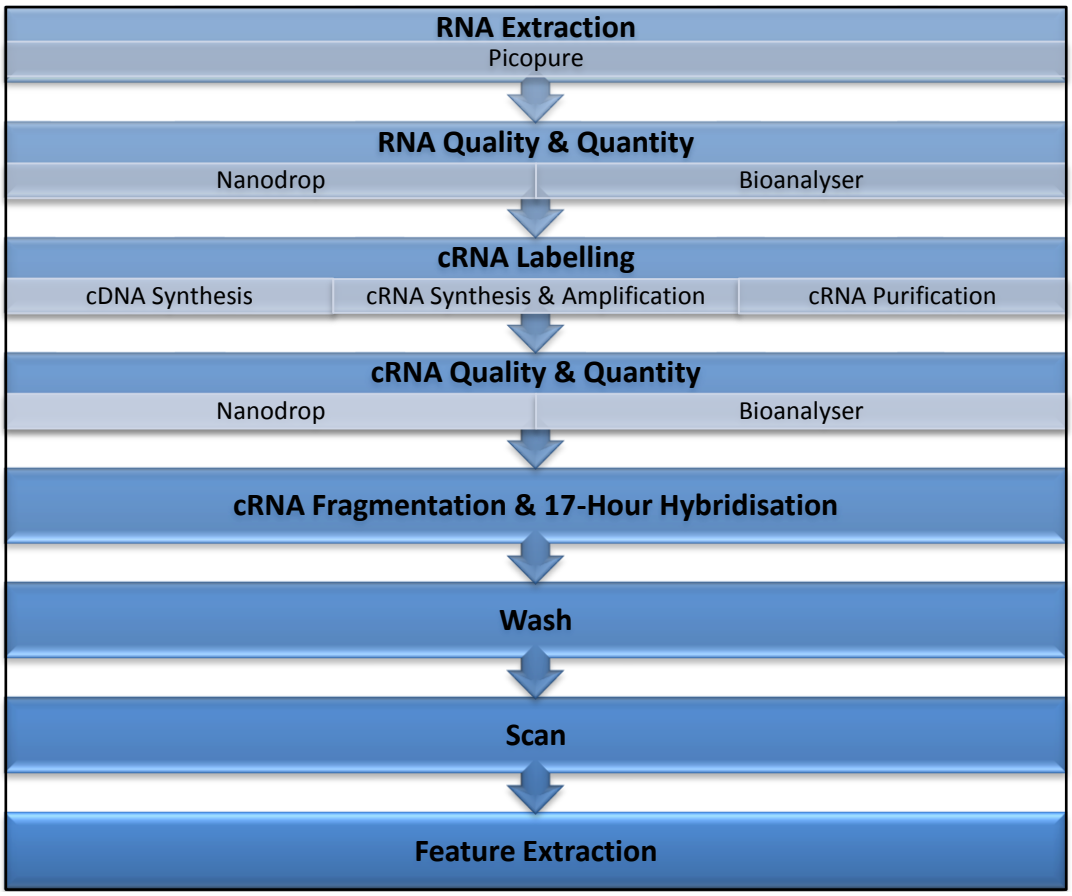


Figure 2.5 Workflow for sample preparation and array processing.
Adapted from Agilent Technologies Two-colour microarray-based gene expression analysis, low input quick amp labelling protocol (2009).

2.2.4.1 *RNA extraction*

RNA was extracted from 3 replicates of pools of 10 adult unexposed *Ae. aegypti* mosquitoes from 4 different strains using the Arcturus® Picopure RNA Extraction Kit (Life Technologies, California, USA), following the manufacturer's protocol. Due to the large size of the adult mosquitoes, replicates were divided into two sets (5 mosquitoes in each set per replicate). Quality and quantity of RNA were assessed by using the Nanodrop ND-1000 (Thermo Scientific, Delaware, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA). Low quality samples were discarded.

2.2.4.2 *cRNA labeling*

100ng of each RNA sample were amplified and labeled using low input Quick Amp labeling kit for 2 colours (Agilent Technologies). The two sets extracted RNA from each replicate were combined before doing the labeling. Each sample was labeled with Cy-3 dye (Cyanine 3-CTP: fluoresces green) and Cy-5 (Cyanine 5-CTP: fluoresces red) dyes in different tubes. After labeling, cRNA was purified using QIAGEN RNeasy mini spin columns (QIAGEN, Hilden, Germany). Labeled RNA quality and quantity were measured by the Nanodrop ND-1000 (Thermo Scientific) and the Agilent 2100 Bioanalzer (Agilent Technologies).

2.2.4.3 Microarray h*ybridization*

cRNA fragmentation and hybridisation were done using the Gene Expression Hybridisation kit (Agilent technologies, UK) following the manufacturer's protocols. For *Ae. aegypti*, microarray hybridizations were performed with the 8 x 15K Agilent *Aedes aegypti* Chip designed by LSTM, where as for *Ae. albopictus* 8x 60k Agilent *Aedes albopictus* chip that was designed by Charles Wondji was used. For each hybridization, 300 ng of labeled cRNA were used. Three biological replicates were tested for each comparison, and dye swapping was also done. Due to the limited number of available arrays, one replicate for each comparison did not have its respective dye swap. Hybridization was conducted for 17 hours at 65°C and 10 RPM. The microarray slides were then washed using the Agilent Microarray Hybridization Kit (Agilent Technologies), following manufacturers' protocol. After hybridization, non-specific probes were washed off with the 'Agilent hybridization kit' according to manufacturer's instructions (Agilent technologies, UK).

2.2.4.4 *Scanning and statistical analysis*

Microarrays were scanned using an Agilent G2205B microarray scanner (Agilent Technologies). The Agilent Feature Extraction software (Agilent Technologies) was employed for spot finding and signal quantification for both Cy-3 and Cy-5 dyes.

Data normalization and statistical analyses were carried out using Genespring GX software (Agilent Technologies). For statistical analysis purposes, transcripts labeled as “present” or “marginal” in 5 out of 6 hybridizations were taken into account. A Student’s t-test with a baseline value of 1 (same transcription level) and corrected for multiple testing according to the methods of Benjamin and Hochberg was used to assess the over or under expression of transcripts. Statistically significant samples were filtered again to generate a list of genes differentially expressed transcripts showing a 2 fold change and a Student’s t-test P value less than 0.01 were considered differentially transcribed between the selected experimental groups

2.2.5 Validation of candidate genes using quantitative real-time PCR (qRT-PCR)

The results obtained from microarray were confirmed by performing qRT-PCR on the genes that were over-expressed (candidate genes). 1µg of total RNA from each of the biological replicates that were used in microarray was used as template for cDNA synthesis that will be explained in section 2.2.6. First, a serial dilution of the cDNA was used to generate standard curves for the housekeeping and candidate genes to assess the efficiency of the PCR as well as the quantitative differences between the samples.

Specific primers were designed to amplify a region of about 100 – 150 base pairs of the coding sequence preferably spanning between two exons for each of the candidate genes. Specific primer designs for candidate genes of *Ae. aegypti* and *Ae. albopictus* will be described in chapters 4 and 5 respectively. All the primer pairs tested had efficiency between 90 and 110%.

The qRT-PCR amplification was conducted using the MX3005 qPCR system (Agilent Technologies). The reaction was done in 20 µl volumes containing: 10 µl Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent), 0.6 µl of each primer (final concentration 600nM), 7.8 µl of nuclease free water and 1 µl template cDNA (either 1/125 or 1/625 dilution depending on the optimization from the serial dilutions). The thermal cycling conditions was conducted in a 3-step program starting with denaturation at 95 °C for 3 min followed by 40 cycles of 10 s at 95 °C and 10 s at 60 °C and a last step of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s. For each sample, three biological replicates and three technical replicates from the standard curve was run simultaneously.

After amplification, the qRT-PCR data was analysed using the MxPro qPCR software (Agilent Technologies). The relative expression and fold changes of the candidate genes were calculated according to the $2^{-\Delta\Delta CT}$ method by combining the PCR efficiency after normalisation with the housekeeping genes (Schmittgen and Livak, 2008).

2.2.6 cDNA synthesis

For making cDNA, RNA was used as template by conducting reverse transcription. 1 µg total RNA was used to produce cDNA. The initial step involves a 13 µl reaction mix containing 1 µl oligo-dT20 (50mM), 1µg RNA in 8 µl nuclease free water, 3 µl nuclease free water and 1 µl dNTP mix (10mM) and incubated at 65 °C for 5 min. Next, 4 µl 5x first strand buffer, 1 µl DTT (0.1M), 1 µl RNase Out and 1.5 µl Superscript III RT was added to the initial reaction and incubated at 25 °C for 5 min followed by 50 °C for 60 min and 70 °C for 15 min. The cDNA product was finally treated with 1 µl RNase H (E. coli) (Invitrogen) and incubated for 20 min at 37 °C to remove RNA. The concentration of cDNA was assessed using Nanodrop ND-1000 (Thermo Scientific).

The final cDNA was then used for quantitative real-time PCR (qRT-PCR) or direct sequencing.

2.2.7 Sequencing of *kdr* gene (intron sequencing)

As mentioned in part 2.2.3.1, three *kdr* mutations has been reported in *Ae. aegypti*. After conducting pyrosequencing, no correlation could be found between the *kdr* F1534C genotype and phenotype of the samples tested. Intron 26 to exon 29 of the voltage gated sodium channel (VGSC) was sequenced to find a correlation between the haplotype of the VGSC gene and the phenotype and also to find other possible mutations. Samples that were used were only from *Ae. aegypti*, and will be described more in Chapter 4.

PCR reactions were carried out in a volume of 15 µl containing 1µl of genomic DNA, 1.5 µl of 10X KAPA Taq buffer A (KAPA Biosystems), 0.12µl of KAPA taq (KAPA Biosystems), 0.12µl dNTP (25µM), 0.75µl $MgCl_2$ (25µM), 0.51 µl of each primer and 10.49 µl of dH_2O. The cycle conditions were 95°C for 5 min and 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. The PCR products were size separated on a 1.5% agarose gel stained with ethidium bromide (0.5µg/µl) and visualised using a gel electrophoresis to confirm the product size. Primer designs are as in Table 2.2.

The samples were purified using the Qiaquick PCR purification kit (Qiagen) and eluted with 30 µl elution buffer and sequenced directly by Microgen, Korea. The sequences were aligned and analysed using Bioedit software.

2.2.8 Sequencing of *kdr* gene (exon sequencing)

To identify other possible mutation that was not detected using the pyrosequencing method, exon 19 to exon 31of the VGSC gene was sequenced. Details of the samples that were used will be described more for both *Ae. aegypti* (Chapter 4) and *Ae. albopictus* (Chapter 5).

PCR reactions were conducted using cDNA. The final volume of the PCR was 15 µl containing 1µl of cDNA, 3 µl of 5X HF buffer A, 0.15µl of Phusion taq , 0.12µl dNTP (25µM), 0.51 µl of each primer and 9.71 µl of dH_2O. The cycle conditions were 98°C for 1 min and 35 cycles of 98°C for 10 s, 63°C (60°C for

Ae. albopictus) for 30 s and 72°C for 1 min and 30 s, followed by a final extension step of 72°C for 10 min. The PCR products visualised using a gel electrophoresis. Primer designs are as in Table 2.2.

The samples were purified using the Qiaquick PCR purification kit (Qiagen) and sequenced directly by Microgen, Korea. The sequences were aligned and analysed using Bioedit software.

Primer Name	Sequence	Product size (base pairs)
In26ex29 F	TCT CAT CTC TCC GAA GAT GCT CTG TA	1026 bp
In26ex29 R	TCC TCC GTC ATG AAC ATT TCC AGT G	
cDNAex19ex31 F	CTT CGA GTG TTC AAG CTA GCG AAA TC	2586 bp
cDNAex19ex31 R	CTG AAA CAG CAG GAT CAT GCT CTG	
AaeAce1 F	ATATATTCTCGACCTCTGACACCG	2562 bp
AaeAce1 R	AAACATGTTACTAGAACTAGCGAGACTA	
AlbAce1 F	GGAGATCCGAGGCCTAATAACC	2098 bp
AlbAce1 R	GAAACGGGTTACTAGAACTAGCGA	

Table 2.2 Primers used for this study.

2.2.9 Genotyping using allele specific PCR

From the sequencing of exon 19 to exon 31, *kdr* 1016 mutation was observed which was previously undetected by using the pyrosequencing method. This mutation could be genotyped by using allele specific PCR method that was described by Saavedra-Rodriguez *et al.* (2007) recording the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Ae. aegypti.*

Since the sequencing of exon 19 to exon 31 showed only the presence of *kdr* 1016, the primers used are only for the detection of *kdr* 1016. Detailed explanation on the samples used will be in Chapter 4.

PCR was performed in a 25 µl volume in 96-well plates (Agilent technologies). Each reaction contained 12.5 µl of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent) 25 pm of each primer, ~100 ng of template DNA, and dH_2O was added to make a final 25 µl volume. PCR wells were covered with Flat Cap Strips (Bio-Rad Laboratories) and placed into MX3005 qPCR system (Agilent Technologies). Thermal cycling conditions were: 95 °C for 12 min (first denature); 39 cycles of 95 °C for 20 s (denature in cycle); 60 °C for 1 min (anneal); 72 °C for 30 s (extension); 72 °C for 5 min (final extension); and ramp from 65 °C to 95 °C at a rate of 0.2 °C/s (melting curve). The data obtained was analysed using the MxPro qPCR software (Agilent Technologies).

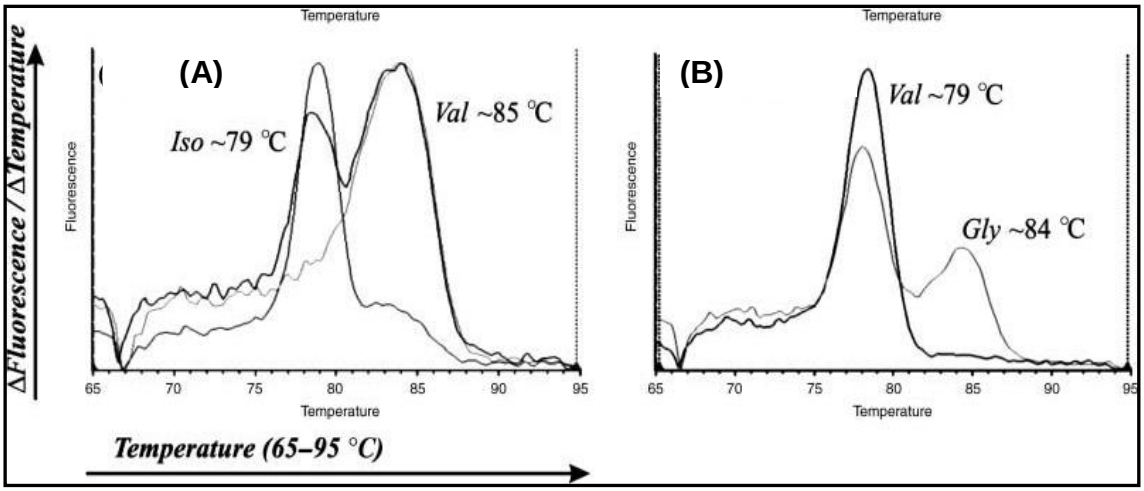


Figure 2.6 Melting curve patterns for genotypes at two single nucleotide polymorphism (SNP). (A) Iso1016Val SNP. (B) Iso1016Gly SNP. Figure from (Saavedra-Rodriguez *et al.*, 2007).

2.2.10 Genotyping of *Ace1* gene

After exposing some mosquitoes to malathion and bendiocarb, resistance phenotype could be identified. To identify whether a mutation was present, the *Ace1* gene was amplified. PCR reactions were conducted using cDNA. The PCR mix was made up containing 1µl of cDNA, 3 µl of 5X HF buffer, 0.15µl of Phusion taq , 0.12µl dNTP (25µM), 0.51 µl of each primer and 9.71 µl of dH_2O. The cycle conditions were 98°C for 1 min and 35 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 1 min and 30 s, followed by 72°C for 10 min.

The PCR products visualised using a gel electrophoresis. Primer designs are as in Table 2.2.

The samples were purified using the Qiaquick PCR purification kit (Qiagen) and sequenced directly by Bioscience. The sequences were aligned and analysed using Bioedit software.

3.0 ASSESSING THE SUSCEPTIBILITY STATUS OF *AEDES AEGYPTI* AND *AEDES ALBOPICTUS IN* MALAYSIA

3.1 Introduction

Aedes aegypti and *Ae. albopictus are* widely distributed throughout Malaysia (Lo and Narimah, 1984, Yap *et al*., 1984). The distribution of these species has started to become overlapped but generally, *Ae. aegypti* can be found in rapidly developing areas with less vegetation whereas *Ae. albopictus* prefers outdoor conditions with more vegetation (Chen *et al.*, 2006). The main control methods in Malaysia are adulticiding with permethrin, deltamethrin and malathion and larviciding with temephos and *Bti (Bacillus thuringiensis israelensis)* (MOH, 2011). Insecticides are widely used in Malaysia not only by the Ministry of Health (MOH) operators but also by private companies and the community to control mosquitoes as well as other household pests (Rohani *et al.*, 2011).

Although resistance has been detected in various *Aedes* populations in Malaysia, the knowledge is still limited as the extent of the resistance as well as its geographical distribution across the country remains unknown.

In this chapter, the resistance pattern of *Ae. aegypti* and *Ae. albopictus* field populations were investigated. Larval bioassays were conducted to assess the resistance towards temephos and adult bioassays were used to test the susceptibility to four main insecticide classes. PBO synergist assays were also conducted to identify the presence of both target site mutations and metabolic resistance mechanisms. In Malaysia, only a handful of studies have been conducted to assess the resistance pattern of *Aedes* sp., but those studies focused only on the major cities such as Kuala Lumpur (Selvi et al., 2010, Chen et al., 2005b) and Penang (Chan *et al.*, 2011) and no nationwide study has been carried out before. Thus, this study aimed to assess the distribution of resistance throughout Malaysia and to establish the extent of resistance towards different classes of insecticide. The information obtained is crucial to implement suitable evidence-based control strategies of both *Aedes* species and overall reducing the number of dengue cases in Malaysia.

3.2 Materials and Methods

3.2.1 Origin of strains

Field collections were performed in residential areas in four states across Malaysia; Penang (Northwest), Kelantan (Northeast), Federal Territory of Kuala Lumpur (Centre) and Johor (South) (Chapter 2, Figure 2.1). In each state, two locations were chosen. All of these areas were dengue endemic areas during the time of sample collection. Details of the locations are in Table 3.1. *Aedes aegypti* New Orleans (NO) colony reared in Liverpool School of Tropical Medicine (LSTM) was used for susceptible laboratory strain. For *Ae. albopictus*, a susceptible laboratory colony from Vector Control Research Unit (VCRU), Penang was used.

State	City	Location	Coordinates
*Penang	Bayan Lepas	Bukit Jambul	05° 20′ 18”N 100° 17′ 01”E
		Permatang Damar Laut	05° 16′ 34”N 100° 16′ 06”E
Federal Territory of Kuala Lumpur	*Kuala Lumpur	Taman Desa Tasik	03° 04' 12"N 101° 42' 59"E
		Sungai Besi	03° 07′ 25”N 101° 42′ 33”E
Kelantan	*Kota Bharu	Bandar Baru Kubang Kerian	06° 06' 17"N 102° 17' 09"E
		Jalan Telipot	06° 06 ′33”N 102° 14 ′42”E
Johor	*Johor Bharu	Taman Century	01° 28' 52"N 103° 45' 43"E
		Taman Gembira	01°31′ 19”N 103° 44′ 42”E

Table 3.1 Details of collection sites. *marks the name given to each strain.

3.2.2 Collection Method

Sample collection was conducted using ovitraps made from 400 ml tin cans which had been painted black to attract mosquitoes (Figure 3.3(a)). Two holes were made at the side of the tin can to act as a drain to remove access water in case it rained. A wooden paddle was placed in the tin can which had been half filled with water (unbaited). A sticker with contact details was fixed onto each ovitrap for residence to contact in case of any query.

Approximately 80 ovitraps were set up in two collection sites in each state. Ovitraps were placed in locations close to human dwellings and also near vegetation to be able to collect both *Aedes* species (Figure 3.2). The traps were collected five days after they had been placed and all the water containing larvae and wooden paddles containing eggs were brought back to the lab at the VCRU, Universiti Sains Malaysia in Penang. During the long car journey back to Penang especially from Johor Bharu (8 hours) and Kota Bharu (6 hours), ample amount of food were given to the larvae to avoid any deaths. The wooden paddles were also ensured to be moist to avoid desiccation of the eggs. The traps were also kept in order to be rinsed out in the lab.

Aedes larvae were also collected from old tyres, flower pots, tree holes and other containers that held water (Figure 3.3). The larvae were collected by pipettes or the water was poured into a secure container.

The eggs and larvae were reared in the labs and morphologically identified and separated at the adult stage. The colonies were blood fed and a few batches of eggs were collected. Both the larval bioassays and adult bioassays were conducted using F1 mosquitoes.

Figure 3.2 Examples of ovitrap placement.

Figure 3.3 Examples of method of collection. (a) Ovitrap (b) Collection of water from ovitraps (c) Larvae collection (d) Tyre (e) Larvae picked from tyre.

3.2.3 Larval Bioassay

Temephos larval bioassays were conducted according to WHO guidelines (WHO, 2005a). Temephos was chosen for this experiment because it is used to control mosquito larvae in the Ministry of Health control programs (MOH, 2011).

Preliminary tests on lab strains were done to determine the range of temephos concentration to be used for the bioassay. For each concentration of Temephos, 4 replicates of 25 larvae were used. From the preliminary test, 8 concentrations were used for *Ae. aegypti*; 0.002ppm, 0.004ppm, 0.006ppm, 0.009ppm, 0.012ppm, 0.015ppm, 0.020ppm, 0.025ppm. 10 concentrations were tested on *Ae. albopictus* larvae; 0.002ppm, 0.004ppm, 0.006ppm, 0.009ppm, 0.012ppm, 0.015ppm, 0.020ppm, 0.025ppm, 0.050ppm, 0.075ppm. Controls were done using ethanol.

Due to lack of samples, larval bioassay was unable to be conducted on *Ae. aegypti* Kota Bharu strain and *Ae. albopictus* Johor Bharu and Kota Bharu strain. The lack of samples were because they were used for adult bioassays and we wanted to reduce the error by ensuring that the larvae used were from the same generation in all the experiments conducted.

3.2.4 Adult Bioassay

Adult bioassays were carried out according to WHO protocol (WHO, 1981). All of the strains were tested in 4 replicates of 25 female mosquitoes against six insecticides according to the recommended concentration by WHO (1998) (Table 3.2). The insecticides used were the pyrethroids permethrin (type I) and deltamethrin (type II), the organochlorine DDT and dieldrin, the carbamate bendiocarb and the organophosphate malathion. Controls were done using papers impregnated with carrier oils only.

For permethrin, bioassay was conducted using the 0.75% insecticide impregnated paper which is the recommended concentration for Anopheline mosquitoes and not the 0.25% impregnated paper which is for *Ae. aegypti*

(WHO, 1998). This was because 0.25 % papers were not available. Many other studies on *Aedes* also used the Anopheles diagnostic dosage of 0.75% (Nazni *et al.*, 2009, Harris *et al.*, 2011, Wan-Norafikah *et al.*, 2013).

Mosquito strains that were highly resistant to permethrin, deltamethrin, DDT and bendiocarb were also tested using insecticide synergist, PBO to assess the involvement of cytochrome P450s in the insecticide resistance mechanisms. Mosquitoes were pre-exposed to 4% PBO for 1 hour before directly exposed to the mentioned insecticides. In parallel, bioassays using only PBO 4% and bioassays exposing the mosquitoes to control papers before transferring to insecticide tubes were done as control.

Unfortunately, due to the low numbers of mosquitoes available, the synergist bioassay was unable to be conducted on *Ae. aegypti* Kota Bharu strain and *Ae. albopictus* Johor Bharu and Kota Bharu strain.

Class	**Insecticide**	**Anophelines**	***Ae. aegypti***	***Cx. quinque-fasciatus***
Organochlorines	DDT	4%	4%	4%
	Dieldrin	4% and 0.4%		
Organophosphates	Fenitrothion	1%		1%
	Malathion	5%	0.8%	5%
Carbamates	Bendiocarb	0.1%		
	Propoxur	0.1%	0.1%	0.1%
Pyrethroids	Alpha-cypermethrin	0.05%		
	Bifenthrin	0.2%		
	Cyfluthrin	0.15%		
	Deltamethrin	0.05%		0.025%
	Etofenprox	0.5%		
	Lambda-cyhalothrin	0.05%	0.03%	0.025%
	Permethrin	0.75%	0.25%	0.25%

Table 3.2 Discriminating concentrations of insecticides for adult mosquitoes. (Adapted from WHO, 1998 and WHO, 2013).

3.2.5 Data Analysis

Percentage mortality was calculated for the number of mosquitoes or larvae that were dead/knockdown 24 hours after exposure. The LC_{50} value for the larval bioassay was calculated using regression-probit analysis (R statistical software). Resistance ratio (RR) was also calculated by using the formula below by comparing against susceptible strain. The susceptible strain for *Ae. aegypti* was from New Orleans (NO) and for *Ae. albopictus* was from Vector Control Research Unit (VCRU).

$$\textbf{Resistance ratio (RR)} = \frac{\textbf{LC}_{50}\textbf{ of Resistant strain}}{\textbf{LC}_{50}\textbf{ of Susceptible strain}}$$

Resistance ratio formula

If the control population showed mortality between 5% and 20%, the percentage mortality was re-calculated using Abbott's formula as shown below.

$$\frac{\textbf{\% test mortality – \% control mortality}}{\textbf{100 - \% control mortality}} \textbf{ X 100}$$

Abbott's formula for corrected mortality

3.3 Results

3.3.1 Larval Bioassay

From this point onwards, abbreviations will be used to name the mosquito strains; New Orleans (NO) and Vector control Research Unit (VCRU) laboratory susceptible strains, Penang (PG), Kuala Lumpur (KL), Johor Bharu (JB) and Kota Bharu (KB) field strains.

The larval bioassay using temephos was conducted in parallel with untreated control batches (ethanol only) and there was no mortality observed in all of the controls tested (*Ae. aegypti* n = 300, *Ae. albopictus* n = 225).

For *Ae. aegypti*, LC_{50} for the PG strain was highest at 0.008ppm followed by KL and JB both with 0.006ppm (Appendix 8.1) . The resistant ratio (RR) for PG increased to 2.0 fold when compared with the susceptible NO strain where as the RR for both KL and JB were 1.5 (Table 3.3). Statistical significance could only be observed between the NO and PG strain.

In the case of *Ae. albopictus* , the LC_{50} for PG and KL strain was 0.020ppm and 0.015ppm respectively (Appendix 8.2). The RR against temephos for PG is 3.3 and 2.5 for KL (Table 3.4). There is a significant difference between the three *Ae. albopictus* strains.

Strain	Sample size	LC_{50},ppm (95% C.I.)	RR
New Orleans	640	0.004 (0.003 – 0.006)[a]	1
Penang	640	0.008 (0.008 – 0.009) [b]	2
Kuala Lumpur	640	0.006 (0.005 – 0.006)	1.5
Johor Bharu	640	0.006 (0.005 – 0.006)	1.5
Kota Bharu	N/A	-	

Table 3.3 LC_{50} and RR from larval bioassays on *Ae. aegypti* strains against NO Laboratory strain, 4 replicates tested for each temephos concentration. C.I.= Confidence Interval. [a,b] Statistically significant using overlapping of confidence interval test.

Strain	Sample size	LC_{50},ppm (95% C.I.)	RR
VCRU	640	0.006 (0.006 – 0.007)	1
Penang	640	0.020 (0.018 – 0.021) [a]	3.3
Kuala Lumpur	640	0.015 (0.014 – 0.016) [b]	2.5
Johor Bharu	N/A	-	-
Kota Bharu	N/A	-	-

Table 3.4 LC_{50} and RR from larval bioassays on *Ae. albopictus* strains against VCRU Laboratory strain, 4 replicates tested for each temephos concentration. C.I.= Confidence Interval. [a, b] Statistically significant using overlapping of confidence interval test.

3.3.2 Adult Bioassay

A baseline test was done for the lab strains of *Ae. aegypti* NO strain and *Ae. albopictus* VCRU strain. *Aedes aegypti* NO strain was fully susceptible to all six insecticides tested. Surprisingly, *Ae. albopictus* VCRU strain showed resistance towards dieldrin with 80% ± 1.4 mortality for males and 73% ± 1.4 mortality for females (Table 3.5). Another lab strain of *Ae. albopictus* from Institute for Medical Research Malaysia (IMR) was also tested, but that strain was resistant to even more insecticide classes such as pyrethroid and also organochlorine. That is the reason VCRU strain was chosen over IMR strain for conducting assays.

Insecticide	% Moratlity ± SD			
	Ae. aegypti		***Ae. albopictus***	
	Males	**Females**	**Males**	**Females**
Permethrin (0.75%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Deltamethrin (0.05%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
DDT (4%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Bendiocarb (0.1%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Malathion (5%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Dieldrin (4%)	100 ± 0	100 ± 0	80 ± 1.4	73 ± 1.4

Table 3.5 Percentage mortality for baseline test of adult bioassays on *Ae. aegypti* NO strain and *Ae. albopictus* VCRU strain (n = 60). SD = Standard deviation.

3.3.2.1 *Aedes aegypti* resistance pattern

For the *Ae. aegypti* field populations, high levels of resistance were observed in both males and females for the type I pyrethroid, permethrin. For the females, strains from PG, KL, JB and KB showed 33%, 1%, 59.2% and 10% mortality respectively. In the males, the highest level of resistance was in the KL strain with 0% mortality followed by KB, PG and JB with 8%, 43% and 72% mortality (Table 3.6, Figure 3.4). For deltamethrin (type II pyrethroid), most of the strains were resistant except for males from JB and KB which showed 100% and 91% mortality respectively. The mortality for females was 72% for PG, 0% for KL, 79% for JB and 82% for KB (Table 3.6, Figure 3.4).

Between the two types of pyrethroids, the field strains had lower mortality towards permethrin when compared to deltamethrin (Table 3.6, Figure 3.4).

All the strains were highly resistant to DDT with JB being the least resistant with 57% mortality for males and 50.4% mortality for females. KL was the most resistant with 0% mortality for both sexes. Penang showed 17% mortality in females and a significantly higher mortality in males with 50% mortality. On the other hand, there was just a slight difference in the mortality between males and females from KB with 8% and 10% mortality respectively (Table 3.6, Figure 3.4).

Very high levels of resistance towards bendiocarb could be observed in all strains except from KB with 84% mortality for males and 91% mortality for females. For strains from PG and KL the mortality in males were lower than the females; (Table 3.6, Figure 3.4).

For malathion, all of the strains were fully susceptible with almost all showing 100% mortality except for KL females (91%), JB males (98%) and JB females (99%) (Table 3.6, Figure 3.4).

Full susceptibility could be seen in all strains when exposed to dieldrin except for a slight resistance in JB strain with 84% mortality in males and 88% mortality in females (Table 3.6, Figure 3.4).

From the PBO synergist assay, *Ae. aegypti* mosquitoes from all three populations (excluding strains from KB due to low sample size) showed a significant increase in percentage of mortality compared to the results of the normal susceptibility assay (Table 3.6, Figure 3.5). For PBO + permethrin and PBO + deltamethrin assay, full recovery with 100% mortality could be seen in both PG and JB strains. In KL strain, males showed a significant increase in mortality when comparing between assays without synergist and with synergist; for permethrin the increase is from 0% mortality to 93% mortality and for deltamethrin the increase is from 9% to 87% mortality. For females the percent mortality was 26% (PBO + permethrin) and 71% (PBO + deltamethrin). The result obtained for KB strain with 5% mortality when

exposed to PBO and permethrin could be disregarded since the number of tested mosquitoes were too low (n=25) (Table 3.6, Figure 3.5).

For synergist plus DDT assays, the mortality for PG strain was 100% in males and 55% in females, for KL strain it was 68% and 35% mortality for males and females respectively and for JB strain it was 97% mortality in males and 80% mortality in females (Table 3.6, Figure 3.5). High level of susceptibility could be seen in all the strains after exposure to PBO and bendiocarb. The mortality for PG, KL and JB males were 95%, 87%, 97% whereas for the females are 65%, 98% and 93% respectively (Table 3.6, Figure 3.5).

3.3.2.2 *Aedes albopictus resistance* pattern

Aedes albopictus field strains, highly susceptible to permethrin with 95.2% to 100% mortality except for KL which was slightly resistant with 81% mortality in males and 87% mortality in females (Table 3.7, Figure 3.6). The same scenario could be seen in the resistance towards deltamethrin with full susceptibility in both sexes for PG and JB strains. Males from KB also showed 100% mortality whereas the females showed 97% mortality. KL strain was slightly less susceptible with 93% and 89% mortality in males and females respectively (Table 3.7, Figure 3.6).

For DDT insecticide a mixed resistance pattern could be observed when comparing all the four strains. The most highly resistant was the KB strain with 4% mortality for males and 14% mortality for females followed by KL strain with 17% and 6% mortality in males and females respectively. JB strain showed a significant difference between both sexes with males that was highly susceptible with 98% mortality and females with a high level of resistance (60% mortality). PG strain on the other hand showed a different pattern with the males being resistant with 79.2% mortality and the females that were highly susceptible with 96.8% mortality (Table 3.7, Figure 3.6).

High resistance level could be observed for bendiocarb in all the field strains except for KB strain with 98.7% and 93% mortality in males and females respectively and also 100% mortality for males from JB. Females from JB

showed 44% mortality when exposed to bendiocarb which was a significant difference to the males of this strain. PG strain was the most resistant with 32% mortality in males and 28.8% mortality in females followed by KL strain with a mortality of 45% for males and 31% for females (Table 3.7, Figure 3.6).

PG strain exposed to malathion showed a similar resistance pattern with DDT where the males (76.8% mortality) were significantly more resistant than the susceptible females (100% mortality). KB strain was fully susceptible for both sexes with 100% mortality 24 hours after exposure. KL and JB strain showed resistance; 84% mortality for males and 76% mortality for females from KL and for KB 82% and 68% mortality for both males and females respectively (Table 3.7, Figure 3.6).

Strains from JB and KB showed a high susceptibility towards dieldrin with 100% mortality in both sexes except for females from JB with 90% mortality. Slight resistance could be seen KL strain with 90% mortality for males and 84% mortality for females. A higher resistance level could be observed in the PG strain after exposure to dieldrin with mortality of 75.2% in males and 40% in females (Table 3.7, Figure 3.6).

For the synergist assay, bioassays were not done for permethrin and deltamethrin due to the fact that the resistance level was low. Also, it should be noted that the test could not be conducted on populations from JB and KB due to limited availability of mosquitoes. After exposure to PBO and DDT, the strain from PG showed a recovery with 97% mortality in males and 99% mortality in females. For KL strain significant increase in mortality when comparing between assays without synergist and with synergist could be observed; for males from 17% mortality to 91% mortality whereas for females from 6% mortality to 52% mortality (Table 3.7, Figure 3.7).

Bioassays conducted using PBO + bendiocarb showed 79% mortality in males and 53% mortality in females from PG. For KL strain, 100% was observed for males whereas the females showed 57% mortality (Table 3.7, Figure 3.7).

INSECTICIDE	% MORTALITY ± SD							
	PENANG		KUALA LUMPUR		JOHOR BHARU		KOTA BHARU	
	Male	Female	Male	Female	Male	Female	Male	Female
PERMETHRIN	43 ± 1.71 (100)	33 ± 1 (100)	0 (100)	1 ± 0.5 (100)	72 ± 3.6 (100)	59.2 ± 1.3 (125)	8 ± 1.6 (100)	10 ± 2.08 (100)
DELTAMETHRIN	77 ± 1.5 (100)	72 ± 2.94 (100)	9 ± 2.6 (100)	0 (100)	100 ± 0 (100)	79 ± 1.7 (100)	91 ± 2.6 (100)	82 ± 1.3 (100)
DDT	50 ± 2.38 (100)	17 ± 1 (100)	0 (100)	0 (100)	57 ± 3.5 (100)	50.4 ± 1.5 (125)	8 ± 2.2 (100)	10 ± 2.1 (100)
BENDIOCARB	27 ± 1.5 (100)	53 ± 1.26 (100)	20 ± 3.6 (100)	44 ± 3.6 (100)	38.4 ± 3.7 (125)	25 ± 1.7 (100)	84 ± 3.7 (100)	91 ± 1 (100)
MALATHION	100 ± 0 (100)	100 ± 0 (100)	100 ± 0 (100)	91 ± 2.2 (100)	98 ± 1 (100)	99 ± 0.5 (100)	100 ± 0 (100)	100 ± 0 (100)
DIELDRIN	100 ± 0 (100)	100 ± 0 (100)	100 ± 0 (100)	100 ± 0 (100)	84 ± 0.8 (100)	88 ± 2 (100)	100 ± 0 (100)	100 ± 0 (100)
PBO - PERMETHRIN	100 ± 0 (100)	100 ± 0 (100)	93 ± 3.5 (100)	26 ± 1.3 (100)	100 ± 0 (100)	100 ± 0 (50)	*	5 ± 0 (25)
PBO - DELTAMETHRIN	100 ± 0 (100)	100 ± 0 (100)	87 ± 3.9 (100)	71 ± 2.6 (100)	100 ± 0 (100)	100 ± 0 (50)	*	*
PBO - DDT	100 ± 0 (100)	55 ± 1 (100)	68 ± 1.4 (100)	35 ± 1.5 (100)	97 ± 1.5 (100)	80 ± 5.2 (100)	*	*
PBO - BENDIOCARB	95 ± 3 (100)	65 ± 2.5 (100)	87 ± 2.6 (100)	98 ± 0.6 (100)	97 ± 1 (100)	93 ± 2.2 (100)	*	*

Table 3.6 Adult bioassay for different strains of *Ae. aegypti* exposed to six insecticides and PBO synergist. In brackets are the numbers of mosquitoes tested (n). * indicates no data has been produced due to low sample size. SD: Standard deviation.

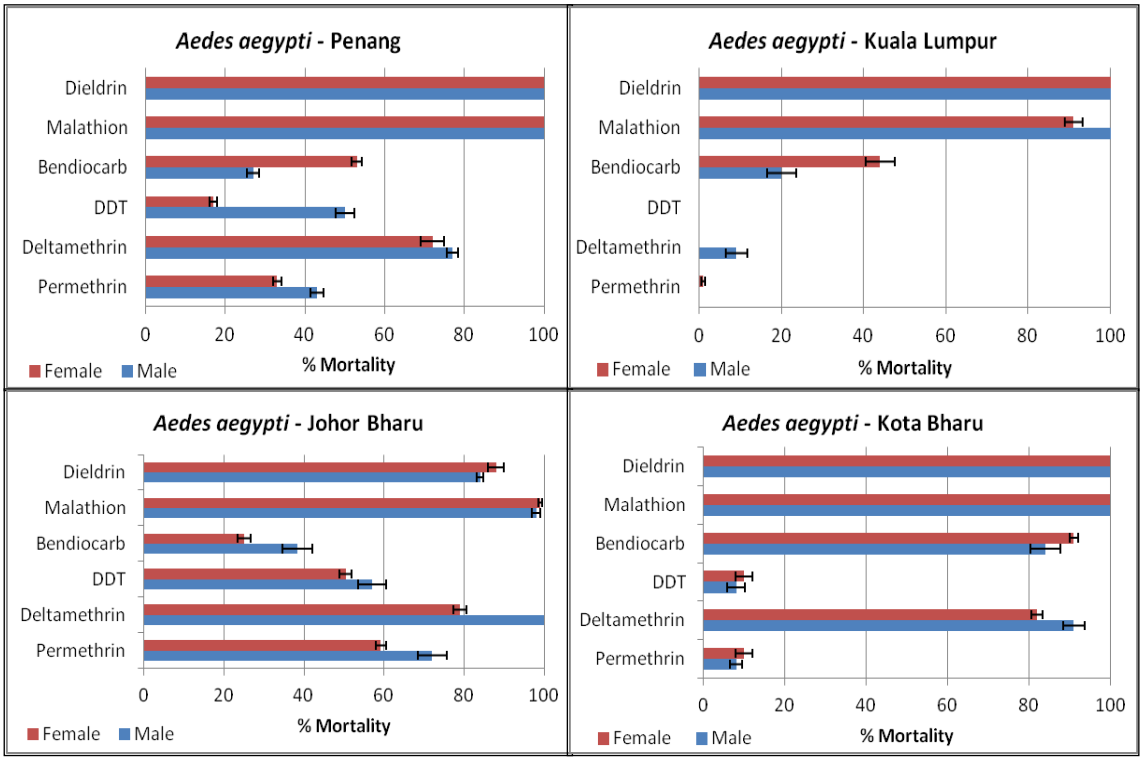


Figure 3.4 Percentage mortality of adult *Ae. aegypti* field strain when exposed to six insecticides. Error bars are standard deviation.

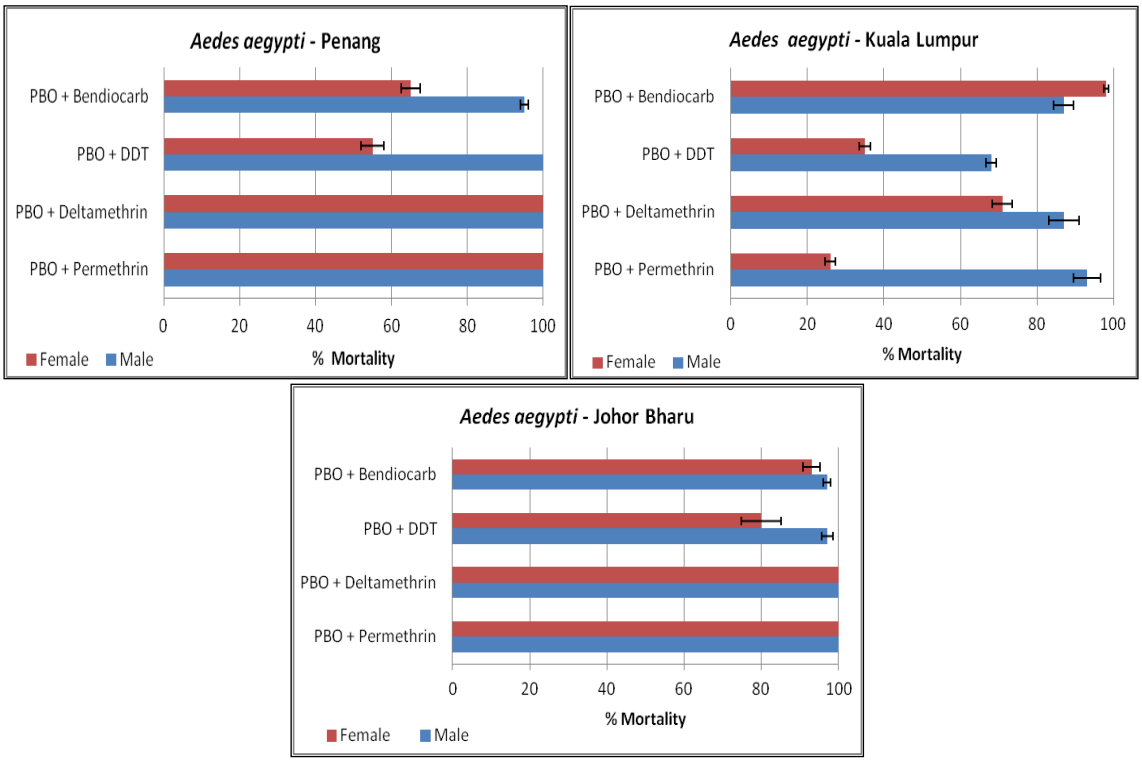


Figure 3.5 Percentage mortality of adult *Ae. aegypti* field strain when exposed to 4% PBO and four insecticides. Error bars are standard deviation.

INSECTICIDE	% MORTALITY ± SD							
	PENANG		KUALA LUMPUR		JOHOR BHARU		KOTA BHARU	
	Male	Female	Male	Female	Male	Female	Male	Female
PERMETHRIN	95.2 ± 1.1 (125)	100 ± 0 (125)	81 ± 6.6 (100)	87 ± 3.9 (100)	100 ± 0 (50)	100 ± 0 (50)	100 ± 0 (100)	100 ± 0 (100)
DELTAMETHRIN	100 ± 0 (125)	100 ± 0 (125)	93 ± 1 (100)	89 ± 1.7 (100)	100 ± 0 (50)	100 ± 0 (50)	100 ± 0 (100)	97 ± 1.5 (100)
DDT	79.2 ± 3.3 (125)	96.8 ± 1.1 (125)	17 ± 3.3 (100)	6 ± 1 (100)	98 ± 0.71 (50)	60 ± 1.41 (50)	4 ± 1 (100)	14 ± 3.7 (100)
BENDIOCARB	32 ± 1.6 (125)	28.8 ± 1.9 (125)	45 ± 3.5 (100)	31 ± 4.4 (100)	100 ± 0 (50)	44 ± 1.41 (50)	98.7 ± 0.6 (100)	93 ± 1.5 (100)
MALATHION	76.8 ± 2.3 (125)	100 ± 0 (125)	84 ± 0.6 (100)	76 ± 0.8 (100)	82 ± 0.71 (50)	68 ± 0 (50)	100 ± 0 (100)	100 ± 0 (100)
DIELDRIN	75.2 ± 3.1 (125)	40 ± 1.4 (125)	90 ± 1.3 (100)	84 ± 2.58 (100)	100 ± 0 (50)	90 ± 0.71 (50)	100 ± 0 (100)	100 ± 0 (100)
PBO - DDT	97 ± 1 (100)	99 ± 0.5 (100)	91 ± 1.7 (100)	52 ± 2.9 (100)	*	*	*	*
PBO - BENDIOCARB	79 ± 1 (100)	53 ± 1.3 (100)	100 ± 0 (100)	57 ± 1.7 (100)	*	*	*	*

Table 3.7 Adult bioassay for different strains of *Ae. albopictus* exposed to six insecticides and PBO synergist. In brackets are the numbers of mosquitoes tested (n). * indicates no data has been produced due to low sample size. SD: Standard deviation.

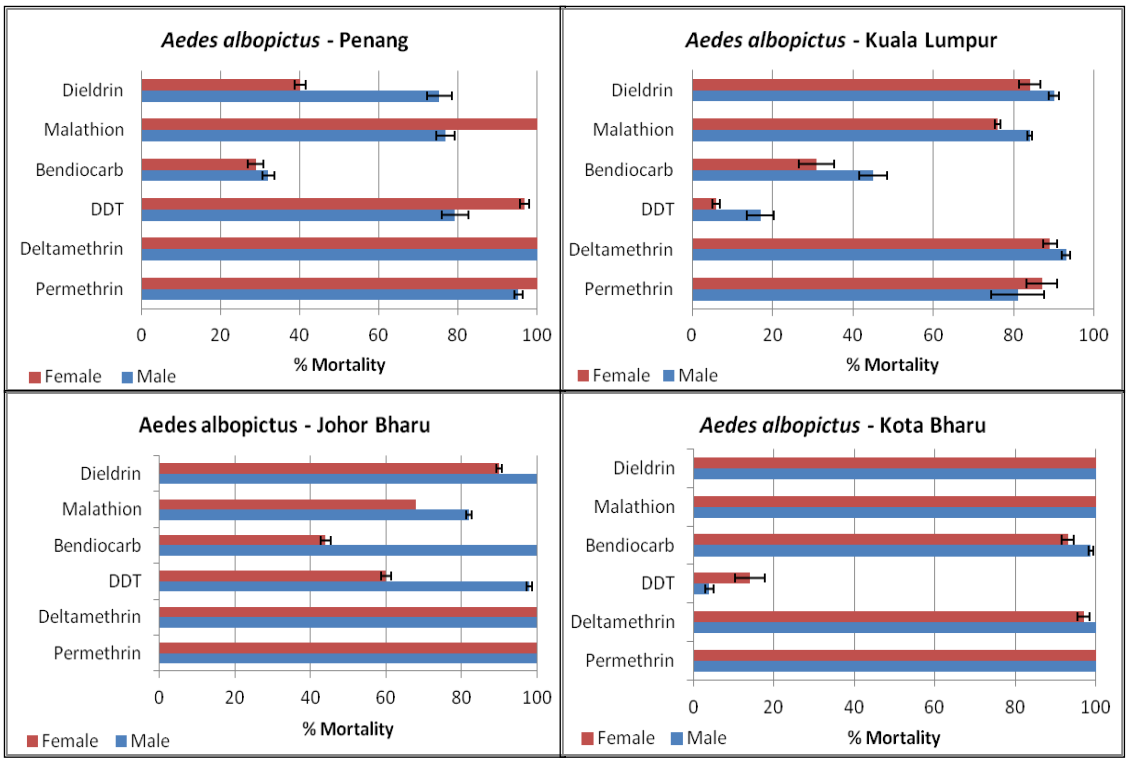


Figure 3.6 Percentage mortality of adult *Ae. albopictus* field strain when exposed to six insecticides. Error bars are standard deviation.

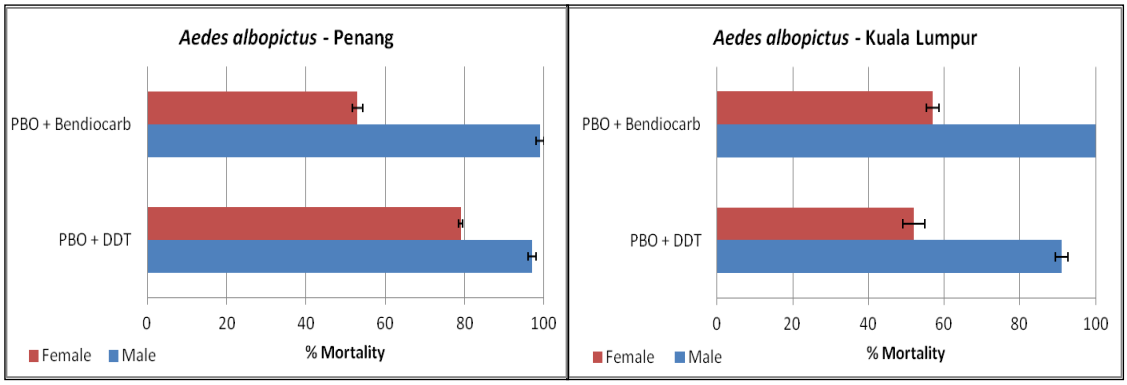


Figure 3.7 Percentage mortality of adult *Ae. albopictus* field strain when exposed to 4%PBO and two insecticides. Error bars are standard deviation.

3.4 Discussion

Previous definition of criteria for insecticide resistance by the WHO (WHO, 1998) states that bioassay mortality of 98% or above indicates insecticide susceptibility and less than 80% reflects insecticide resistance. For 80-97% mortality, it suggests that there is a possibility for resistance which should be confirmed with further bioassays. According to the new revised definition set by WHO (2013a), 90% mortality is already a confirmation of resistance and populations with mortality between 90 – 97% have to confirm the resistance by conducting more bioassay and/or performing molecular assays to identify the resistance mechanism (WHO, 2013a). With regards to these new criteria, this study has shown different levels of resistance between the *Aedes* species and also between the different geographical strains tested.

Temephos or 0,0,0'0'-tetramethyl-0,0'-thiodiphenylene phosphorothiorate is an organophosphate which has low mammalian toxicity and is not harmful when used in operational dosage (Chen *et al.*, 2005a). In Malaysia temephos (Abate®) was first introduced in 1973 and widely used in 1998 during the worldwide pandemic (Ang and Satwant, 2001). The presence of temephos resistance in the field population could be due to the fact that it has been used to control *Aedes* species since 1973 (Chen *et al.*, 2005a). The recommended dosage of temephos (Abate®) for operational purposes in Malaysia is 1mg/L which is about 83 folds higher than the diagnostic dose recommended by WHO. Chen *et al.*, (2005b) reports that 100% mortality could be observed in *Ae. aegypti* strain from KL after 24 hours of exposure to the field dose.

From the larval bioassay, there was not a high level of resistance to the temephos insecticide. *Aedes aegypti* showed a lower value of resistance ratio when compared to *Ae. albopictus* (Table 3.3 and 3.4). For both *Aedes* species, PG strain was the most resistant and out of the two species *Ae. albopictus* seemed to be slightly more resistant with a higher RR value. However, the RR value could not be the sole indicator of resistance when comparing the two species since the susceptible laboratory strains was different. For *Ae. aegypti* the susceptible strain was from New Orleans which had a totally different genetic background from the field strains, hence the

genetic variation could play a role in the resistance. Nevertheless, direct comparison of the LC_{50} values, also confirmed that *Ae. albopictus* populations from Malaysia are more resistant to temephos than *Ae. aegypti.* Even though *Ae. aegypti* is endophilic and most likely to be in contact with temephos containing water in their breeding site, it was unusual to see that they were the less resistant between the two species. However, due to the overlapping of breeding sites, it could be possible that *Ae. albopictus* was more in contact with temephos. Temephos resistance in *Ae. aegypti* larvae had also been reported in field strain from Thailand (Paeporn *et al.*, 2004), Cambodia (Polson *et al.*, 2001), and Brazil (Lima et al., 2011a). In *Ae. albopictus,* temephos resistance was observed in field strains from Thailand (Ponlawat *et al.*, 2005).

Overall, *Ae. aegypti* were more resistant to pyrethroid for both permethrin and deltamethrin compared to *Ae. albopictus*. This could be due to the fact that *Ae. albopictus* has a broader range of breeding sites and *Ae. aegypti* are a more urban species which is closer to human dwellings and are exposed to more insecticide either during fogging by the MOH with water-based pyrethroid such as Resigen and Aqua-resigen (Ang and Satwant, 2001, Rohani *et al*., 2011) or they are more prone to household insecticide exposure (Chen *et al.*, 2005b).

There was also a difference in the resistance when comparing the two types of pyrethroid. In both *Aedes* species, there were more mortality towards permethrin than deltamethrin. This could be due to the fact that they were more exposed to permethrin because it was the insecticide of choice for thermal fogging and ultra low volume spraying in vector control programs (Rohani *et al.*, 2011). Something that should be noted is that for permethrin, the dosage that was used was 3 fold higher (0.75%) than the recommended dose for *Aedes* which was 0.25% (WHO, 1998).

In both *Aedes* species, high DDT resistance was observed. Resistance to DDT could be due to the past usage of this insecticide to control *Ae. aegypti* in Malaysia. Due to its persistent organic nature, DDT it is not easily broken down and stays in the environment even though the usage has been stopped

in 1957 and replaced with dieldrin (Nazni *et al.*, 2009). Studies had reported that strains that were resistant to DDT could develop cross resistance to pyrethroids and vice versa (Nazni *et al.*, 2009). Cross resistance of permethrin and DDT was recorded in urban strain of *Ae. albopictus* in Kuala Lumpur (Selvi *et al.*, 2010).

Aedes aegypti is more susceptible towards malathion compared to *Ae. albopictus*. Before 1996, malathion was heavily used in control programs. But since complaints by the community stating that they did not prefer fogging inside their houses due to malathion being diesel-solvent which left oil residues on floors and wall of houses as well as emitting bad odour the MOH had changed the control to pyrethroids (Ang and Satwant, 2001). In some instances malathion was still used for fogging (personal information obtained from an officer at MOH). The ongoing usage of malathion may have contributed to the slight resistance observed in four field strains.

Dieldrin was used in agriculture and had been banned as a vector control insecticide due to environmental and human health concerns. The chemical compound is similar to DDT which is a photostable component and is hard to breakdown in the environment. In Malaysia it was used to replace DDT in 1980 and was banned in 1994 (http://www.esd.worldbank). Some reports show that dieldrin is still used as an agricultural insecticide illegally. From the adult bioassay conducted PG, KL and JB *Ae. albopictus* strains showed resistance to dieldrin whereas full susceptibility was seen in *Ae. aegypti* populations except for a moderate resistance in JB strain. This could be due to the nature of *Ae. albopictus* with breeding sites near vegetation where they may come in contact with dieldrin.

Form the PBO synergist bioassays, recovery in the percent mortality could be observed in both *Aedes* species. This indicates that the resistance towards permethrin, deltamethrin, DDT and bendiocarb could be caused by metabolic resistance by cyctochrome P450s monooxigenase. However, the role of target site insensitivity resistance mainly knockdown (*kdr*) resistance should not be disregarded. Previous studies on *Ae. albopictus* from Kuala Lumpur has also shown increase mortality after exposure to PBO + permethrin

compared to permethrin only bioassays (Wan-Norafikah *et al.*, 2013). But no resistance mechanisms were investigated.

In some locations *Ae. aegypti* and *Ae. albopictus* were found to share the same breeding container when collection was done. However, different levels of resistance were observed in both of the species and different patterns were observed in different strains. Variation exists between different geographic strains when exposed to insecticides (Selvi *et al.*, 2010). From vector surveillance conducted in Pahang, Malaysia by Norzahira *et el*. (2011), both *Aedes* species could be observed in ovitraps that were placed indoors and outdoors. This concludes that mixed breeding sites are occurring in both of these dengue vectors.

Although many studies have been conducted throughout the world to assess the susceptibility of field mosquitoes, the exact threshold of resistance level that would cause failure of control programs and the outbreak of diseases has not been established. Even though a mosquito population has been reported as resistant, it does not mean that the control program has failed. Other confounding factors could also lead to the failure of control programs such as the emergence of secondary vectors. How rapidly an insecticide becomes ineffective in controlling mosquito vectors depend on the selection pressure of resistance which is determined by frequency and duration of insecticide usage as well as the dosage used (Chen *et al.*, 2005b, Ranson *et al.*, 2008).

This study has shown that both *Aedes* species from all four field populations in Malaysia shows a different susceptibility pattern. This data is important and the underlying mechanism that causes these different resistance patterns has to be explored. The next chapters explain the resistance mechanisms that were detected in *Ae. aegypti* and *Ae. albopictus* populations.

4.0 INSECTICIDE RESISTANCE MECHANISM IN *AEDES AEGYPTI* IN MALAYSIA

4.1 Introduction

There are four major classes of insecticide which are pyrethroids, organochlorines, organophosphates and carbamates. Organochlorines are banned for the use in Public Health but there is an exception where DDT is still used for insecticide residual spraying (IRS) for the control of malaria vectors notably in Africa. The most commonly used insecticides in vector control programs are pyrethroids and organophosphates. These insecticides have two modes of action and the pressure from the use of insecticides has caused resistance to develop in all of these groups of insecticides which is widespread in mosquitoes (Ranson *et al.*, 2010).

Target site resistance is caused by mutations in target genes such as voltage gated sodium channel (VGSC) which causes knockdown (*kdr*) resistance, mutation at the *Ace-1* gene acetylcholinesterase (Ache) and GABA receptors (Sathantriphop *et al.*, 2006). The most important target site resistance is *kdr* as it confers resistance to both pyrethroid and DDT which are key for vector control. *Kdr* occurs as a result of a change in the affinity between the insecticides and their binding sites, because of mutations in the sodium channel (Davies *et al.*, 2007) especially after 'selection' to pyrethroids and DDT.

The most common *kdr* mutation that occurs in several insects which causes a leucine to phenylalanine (L1014F) substitution in the S6 hydrophobic segment of domain II in the voltage gated sodium channel (Williamson *et al.*, 1996) (Davies *et al.*, 2007) which is widespread in West Africa in *An. gambiae* (Czeher *et al.*, 2008). A second single point mutation in the same codon results in a leucine to serine (L1014S) amino acid change which confers a slightly different cross-resistance spectrum, with lower pyrethroid and higher DDT resistance (Ranson *et al.*, 2000) could be found in *An. gambiae* from East Africa (Hemingway and Ranson, 2000, Ranson et al., 2000) and *Cx. pipiens* (Martinez-Torres *et al.*, 1999). Another *kdr* mutation which is the

super-*kdr* mechanism involves a combination of L1014F mutation and another secondary substitution further upstream (Williamson *et al.*, 1996). This mutation causes resistance only in houseflies and confers a high level of pyrethroid resistance (Williamson *et al.*, 1996) but is ineffective against DDT (Usherwood *et al.*, 2005).

These classic 1014 kdr mutations have not been reported for *Ae. aegypti* but other kdr mutations have been described in resistant populations (Brengues *et al.*, 2003, Saavedra-Rodriguez *et al.*, 2007, Harris *et al*., 2010). Four Amino acid substitutions at variable sites are found in the domain II S4 to II S6 region (residues 923, 982, 1011 and 1016) and the fifth is located in the linker between the S5 and S6 region of domain IV at residue 1763 (Brengues *et al.*, 2003, Saavedra-Rodriguez *et al.*, 2007).

The V1016I and I1011M substitutions have been linked to resistance to pyrethroids by comparing allele frequencies in susceptible and resistant populations (Martins *et al.*, 2009, Saavedra-Rodriguez *et al.,* 2007).The V1016I substitution is widespread across Latin America (Saavedra-Rodriguez *et al.*, 2007). An alternative mutation at this residue, V1016G, has previously only been reported in Asian populations of *Ae. aegypti.* The two alternative substitutions at 1011 residue; I1011M and I1011V, have both been reported in Latin America (Rajatileka *et al*., 2008, Saavedra-Rodriguez *et al.*, 2007). It was recently reported that a mutation at domain III S6 which substitute phenylalanine to cysteine in *Ae. aegypti* (F1534C) was also the cause for DDT and pyrethroid resistance (Harris *et al.*, 2010, Yanola *et al*., 2011). This mutation was found in Thailand (Yanola *et al.*, 2011), Vietnam (Kawada *et al.*, 2009) and Cayman Island (Harris *et al.*, 2010). and in *Ae. albopictus* in Singapore (Kasai *et al.*, 2011).

Insensitivity to Acetylcholinesterase (*Ache*) is responsible for organophosphate and carbamate resistance (Hemingway, 2000). Mosquito genome contains two *Ace* genes but only *Ace-1* confers resistance (Hemingway, 2000, Mori *et al.*, 2007). Mutation in this gene confers resistance notable the G119S commonly found in *An. gambiae* and *Cx.*

quinquefasciatus. In wild type population of *Ae. aegypti*, no *Ace-1* mutation has been reported so far.

Another important resistance mechanism is the metabolic resistance through up-regulation of detoxification genes. Mosquitoes have a complex detoxification system composed of enzymes with a range of activity which is able to metabolize xenobiotics. Three large multi-gene families are responsible for insecticide resistance in mosquitoes, and they are the monooxygenase (cytochrome P450s), glutathione S-transferases (GSTs) and carboxylesterases (COEs) (Li *et al*., 2007, Perry *et al.*, 2011). All of these detoxifying enzymes transform potentially damaging molecules into other less toxic forms by biochemical processes. Enzymes can act generally or specifically on an insecticide. Metabolic resistance is a result of point-mutations affecting protein activity (e.g. change in binding affinity or an altered substrate specificity) or via mutations in cis/trans regulatory loci of these three enzyme families. This resistant phenotype is characterized by overproduction of detoxification enzymes or increasing of its specificity and kinetics (Perry *et al.*, 2011).

Elevated levels of cytochrome P450s are responsible in the resistance towards pyrethroids, carbamates and organophosphates (Hemingway, 2000, Strode *et al.*, 2008, Marcombe *et al.*, 2009). *Ae. aegypti* has more P450 genes compared to other mosquito species except *Culex quinquefasciatus* (Arensburger *et al.*, 2010). As mentioned in Chapter 1, the CYP9 family is large in this species. Microarray studies have shown that the CYP6 and CYP9 family are involved in pyrethroid and organophosphate resistance in *Ae. aegypti* (Strode *et al*., 2008, Marcombe *et al*., 2009).

This chapter focuses on the molecular basis of insecticide resistance in Malaysian populations of *Ae. aegypti* mosquitoes. The presence of both target site resistance and metabolic resistance mechanisms in field populations of *A. aegypti* are explored in this study. For target resistance, the mechanisms that are explored are *kdr* resistance, possible *Ace-1* mutation present and also the presence of genes conferring metabolic resistance. As mentioned in the previous chapters, the vector control program in Malaysia mainly uses

pyrethroids (permethrin, S-bioallethrin and cyphenothrin) and organophosphate (malathion and fenitrothion) for ULV and thermal space sprays and Vectobac® as well as Abate® for larviciding during the outbreak of dengue (Ministry of Health Malaysia, personal communication). Unfortunately, the cases of dengue still rise partly due to the development of insecticide resistance. Research on resistance has been conducted in Malaysia by accessing the susceptibility of mosquitoes and only using biochemical assays. However, my study aims to understand at the molecular level the possible resistance mechanisms that are present in *Ae. aegypti* populations across Malaysia. By understanding the molecular basis of resistance (target site and metabolic resistance), better control measures could be implemented in the vector control program by the Health Ministry.

4.2 Materials and Methods

4.2.1 Mosquito samples

Details of field collections, rearing conditions and susceptibility bioassays are described in Chapter 2 and 3. Mosquitoes obtained from the bioassays are used for the molecular experiments described in this chapter.

4.2.2 Genomic DNA extraction and species identification

Genomic DNA was extracted from 50 individual F_0 field mosquitoes for *Ae. aegypti* from Penang (PG), Kuala Lumpur (KL), Johor Bharu (JB) and Kota Bharu (KB) to confirm the species of the samples using the species identification PCR protocol as presented in Chapter 2 (Section 2.2).

Genomic DNA was also extracted from 25 individual females for the *Ae. aegypti* mosquitoes that were used for bioassays from all four strains. Only DNA from mosquitoes exposed to permethrin, deltamethrin and DDT were extracted. The whole mosquitoes were used for dead samples and only legs and wings were used for alive samples after exposure to insecticides. 30 F_0

field mosquitoes and 25 alive and 25 dead (if there are 25 samples available) F_2 generation mosquito samples were used for detection of k*dr* mutation.

4.2.3 Genotyping for target site resistance

Several methods were used to genotype the presence of target site resistance (*kdr* and A*ce-1* mutations) in *Ae. aegypti*. The methods used were pyrosequencing, direct sequencing of the voltage gated sodium channel (VGSC) gene, genotyping using allele specific PCR and genotyping of the *Ace-1* gene.

4.2.3.1 Genotyping of known *kdr* mutations using pyrosequencing

As mentioned in section 2.2.3.1, from past literature it was observed that there are three *kdr* mutations in *Ae. aegypti* which are in Exon 20 [I1011V (or M)] (Brengues *et al.*, 2003), Exon 21 [V1016I (or G)] (Saavedra-Rodriguez *et al.*, 2007) and Exon 31 [F1534] (Harris *et al.*, 2010). From bioassays conducted, result shows that the samples are resistant to pyrethroid and DDT. This shows that the resistance could possibly be caused by *kdr* mutations. Hence, all four field populations of *Ae. aegypti* were sequenced using the pyrosequencing method to detect the presence of these *kdr* mutations.

Preliminary detection was conducted on 30 F_0 samples to assess the possible presence of these mutations and establish the frequency and geographical distribution of the *kdr* mutations in each population. Subsequently, the F_2 generation mosquitoes were tested in order to assess the correlation of the genotype with the resistance phenotype. The numbers of samples used are as show in Table 4.1. In some populations tested, a few susceptible samples were used due to the high resistance prevalence.

Three primer sets to detect three different *kdr* mutation; *kdr*1011, *kdr*1016 and *kdr*1534 were used (Table 2.1). The pyrosequencing experiment was done according to the protocol described in Chapter 2. The results were then analysed by manually looking at the peaks that represents nucleotides

conferring *kdr* genotype in the pyrograms (Figure 4.4). Test for genotype: phenotype association and test for Hardy Weinberg equilibrium was done using Chi square test (Kirkman, 1996) as conducted by Wondji *et al.* (2008a). Association between resistance phenotypes and the genotypes of the resistance mutation was assessed by estimating the odds ratios and the statistical significance based on the Fisher exact probability test.

Strain	Insecticide exposed	Number of samples	
		Alive	Dead
Penang	Permethrin	25	8
	Deltamethrin	25	25
	DDT	25	0
Kuala Lumpur	Permethrin	25	1
	Deltamethrin	25	0
	DDT	25	0
Johor Bharu	Permethrin	25	11
	Deltamethrin	25	25
	DDT	25	2
Kota Bharu	Permethrin	25	10
	Deltamethrin	18	25
	DDT	25	10

Table 4.1 Samples used for pyrosequencing.

4.2.3.2 Sequencing of the voltage gated sodium channel (VGSC)

To further assess the correlation between resistance phenotype and the F1534C mutation on the voltage gated sodium channel, fragment of this gene spanning the 1534 mutation was sequenced and its polymorphism pattern analysed in correlation of resistance phenotypes. Sequencing of the VGSC was done to assess correlation between haplotypes of this gene (particular haplotypes around the F1534C mutation) and resistance phenotype.

A part of the VGSC; from intron 26 to exon 29 was amplified using In26ex29 primers (Table 2.2) and was done according to the conditions described in section 2.2.8. Genomic DNA from five alive and five dead samples after exposure to permethrin from PG, KL. JB and KB was used. Only dead samples were sequenced for KB due to unsuccessful amplifications of the alive samples. For the case of dead samples from KL, only 1 dead mosquito sample after exposure to permethrin was used since there was only 1 susceptible sample (Table 4.2).

Strain	Insecticide exposed	Number of samples	
		Alive	Dead
Penang	Permethrin	6	5
Kuala Lumpur	Permethrin	4	1
Johor Bharu	Permethrin	5	5
Kota Bharu	Permethrin	0	5

Table 4.2 Samples used for sequencing of intron 26 to exon 29 of the VGSC.

4.2.3.3 Search of new *kdr* mutations through sequencing of the voltage gated sodium channel (VGSC)

Exon 19 to exon 31 of the VGSC was also amplified and sequenced in 3 control (non-exposed to insecticide) samples from all four populations to search for possible new mutations. Because of the high resistance levels to DDT and pyrethroids, these samples are mostly also resistant. cDNA was used in the amplification using primers cDNAex19ex31 and according to the method described in section 2.2.8. CDNA was used to reduce contamination from gDNA. The cDNA obtained was from RNA extractions of pools of 10 adult mosquitoes as described in Chapter 2. The primers used spans the regions of only exon 19 to 31 because most mutations associated with *kdr* are always found in this area as these exons span key catalytic areas of the VGSC (Brengues *et al.*, 2003, Davies *et al*., 2007, Saavedra-Rodriguez *et al.*, 2007, Harris *et al.*, 2010).

All the amplified samples were sent for sequencing directly to Microgen (South Korea) and the sequences obtained were aligned using ClustalW (Thompson *et al.*, 1994) procedure implemented in Bioedit software. A mutation leading to an amino acid change (non-synonymous) or not (synonymous) was assessed by using dnaSP software version 5. Sequences were confirmed by conducting BlastX in NCBI.

4.2.3.4 Genotyping using allele specific PCR

To further genotype the V1016G *kdr* mutation after failure by pyrosequencing, the allele-specific PCR also used (Saavedra-Rodriguez *et al.*, 2007). Genomic DNA from 48 F_0 field samples and alive and dead mosquitoes after exposure to permethrin, deltamethrin and DDT from all four strains (Table 4.3) was used to conduct this experiment. The protocol that was used is described in section 2.2.9. The genotypes were determined using the two allele-specific forward primers and the reverse (Table 4.4). To discriminate between the amplification products (either susceptible or resistant genotype), the melting curve was observed. The data obtained was analysed using the MxPro qPCR software (Agilent Technologies).

4.2.3.5 Sequencing of *Ace-1* gene

Due to the detection of both carbamate (bendiocarb) and organophosphate (malathion) resistance throughout Malaysia, the *Ace-1* cDNA was amplified and sequenced to detect potential resistance mutations associated with both resistances. cDNA from PG, KL and JB was amplified for the *Ace-1* gene using the AaeAce1 primers (Table 2.2) and methods described in section 2.2.10. The PCR product was sent for direct sequencing was done by Source BioScience LifeSciences, UK and the data obtained was analysed using Bioedit software.

Strain	Insecticide exposed	Number of samples	
		Alive	Dead
Penang	Permethrin	25	8
	Deltamethrin	25	25
	DDT	25	0
Kuala Lumpur	Permethrin	25	1
	Deltamethrin	25	0
	DDT	25	0
Johor Bharu	Permethrin	25	11
	Deltamethrin	25	24
	DDT	25	2
Kota Bharu	Permethrin	25	10
	Deltamethrin	18	25
	DDT	25	10

Table 4.3 Samples used for allele specific PCR genotyping for V1016G kdr mutation.

Primers	Sequences	Product size (bp)
Glyl016f	5′-ACCGACAAATTGTTTCCC-3′	
Vall016r	5′-[short tail]AGCAAGGCTAAGAAAAGGTTAATTA-3′	60
Glyl016r	5′-[long tail] AGCAAGGCTAAGAAAAGGTTAACTC-3′	80

Table 4.4 Sequences for primers used for allele specific PCR genotyping for V1016G kdr mutation. The sequence for short tail is [GCGGGC] and long tail is [GCGGGCAGGGCGGCGGGGGCGGGGCC].

4.2.4 Investigating metabolic resistance using microarray

Microarray experiment was conducted to identify the genes potentially associated with the metabolic resistance observed in *Ae. aegypti* populations across Malaysia. A genome-wide transcription profiling was carried out to investigate the differential expression profiles of the populations in comparison to a susceptible strain (New Orleans). The samples used were RNA from 3 replicates of pools of 10 adult unexposed mosquitoes from all four strains. Only samples from PG, KL and KB were used in the microarray. Samples from JB were omitted from the microarray experiment due to the population having similar resistance profile to PG (but at a lower level) and therefore possibly conferred by similar resistance mechanisms (Table 3.6). However, samples from JB were used in the validation of the candidate genes through qRT-PCR. RNA from the susceptible NO strain was also used.

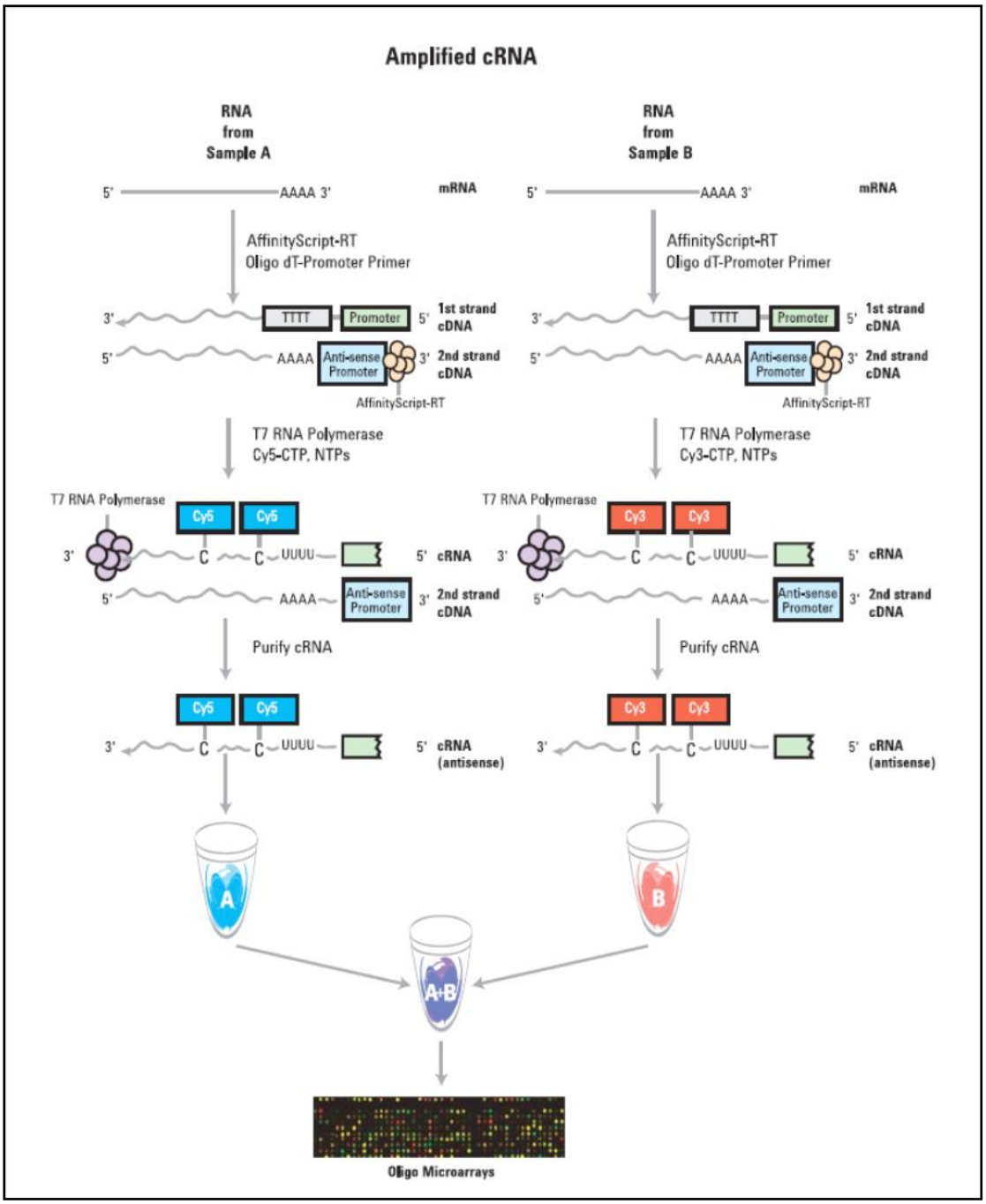


Figure 4.1 Schematic of RNA amplification and labelling process using the Agilent Low Input Quick Amp labelling kit.
Adapted from Agilent Technologies Two-colour microarray-based gene expression analysis, low input quick amp labelling protocol (2009).

4.2.4.1 Microarray design

The microarray hybridization of *Ae. aegypti* samples were done using the 8 x 15k Agilent *Aedes aegypti* chip containing eight replicated arrays of 60-mers oligo-probes representing more than 14,320 different *Ae. aegypti* transcripts from AaegL1.2 Vectorbase annotation and several control probes. This 8 x 15k microarray enables a high coverage across the whole genome (Poupardin *et al.*, 2012) and at the same time reduces cost and increases through-put compared to only using the previous *Aedes* Detox chip which includes only 204 detoxification genes probes used by Strode *et al.* (2008).

In this microarray, the cRNA from samples were reciprocally hybridized against each other in a control vs. susceptible design for all three locations. The three different comparisons were made: New Orleans (NO) susceptible lab strain vs. PG, NO vs. KL and NO vs. KB (control/non-exposed vs. susceptible) (Figure 4.2). Three biological replicates were tested for each comparison, and dye swapping was also done (samples labeled with Cy-3 and Cy-5 dye). A total of five replicates instead of six were used and this approach has been shown valid to detect the resistance genes (Riveron et al., 2013, Kwiatkowska et al., 2013) and also to be cost-effective.

The protocol for RNA extraction, cRNA labelling (Figure 4.1), microarray hybridization, scanning and statistical analysis is discussed in Chapter 2.

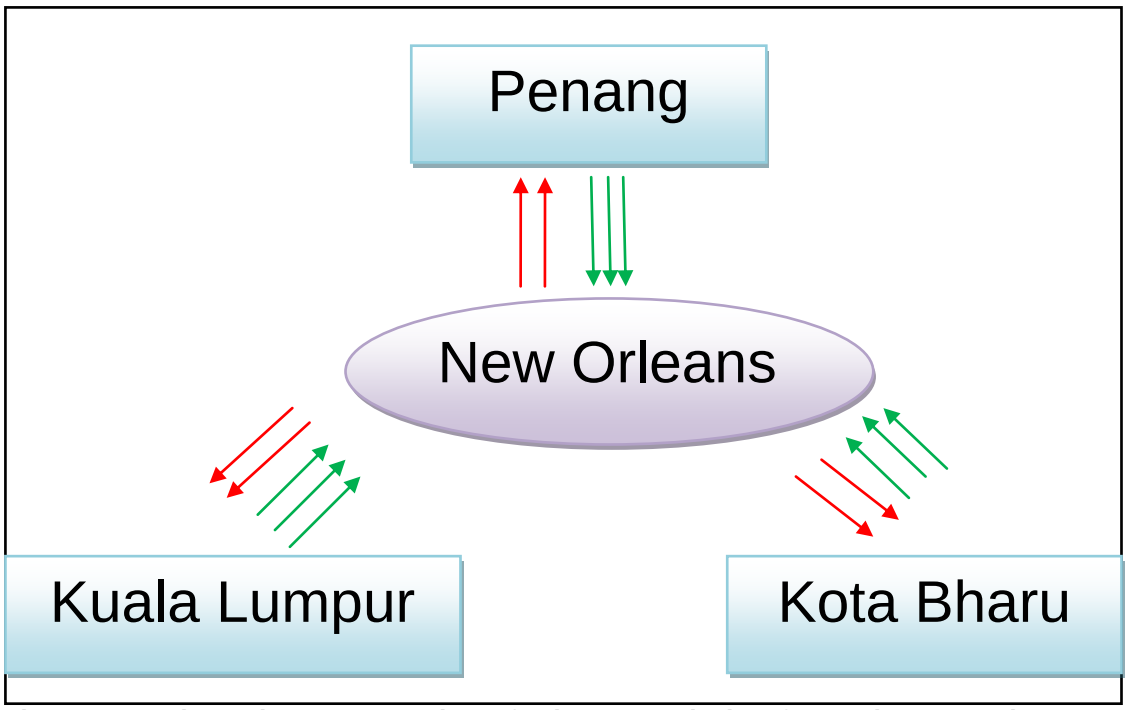


Figure 4.2 Schematic representation of microarray design for *Aedes aegypti*. Green arrows refer to Cy-3 dye and red arrows refer to Cy-5 dye.

The arrays were scanned using an Agilent G2205B microarray scanner (Agilent Technologies, UK) using the default settings following the Agilent Microarray scanner System User Manual (v 7.0).

High (100% photo-multiplier tube (PMT)) and low (10% PMT) extended dynamic range (XDR) scan images were combined and extracted using the Agilent Feature Extraction (FE) software GE2_105_Jan09 (Agilent Technologies, UK) and the custom array grid template (028498_D_F_20100519.XML). Quality control (QC) reports were consulted to give an indication of array quality. A QC score of 11/11 indicates that all 11 main array parameters were passed (Appendix 8.4). QC parameters include signals from spike-in controls, spatial distribution of outliers and signals from non-control spots. A score between 8 and 11 is good and usable and anything below 8 leads to the array having to be repeated.

4.2.4.2 Microarray data analysis and enrichment analysis

Genespring GX version 12 software (Agilent Technologies, UK) was used for statistical analysis of the data obtained from microarray. Mean expression ratios were submitted to a t-test against zero with a multiple testing correction (Benjamini-Hochberg false discovery rate). Genes showing both t-test and p-values less than 0.01 (or 0.05) and a fold change value of 2 were considered significantly differentially transcribed between the two strains compared.

Genes or entities that were considered as significantly differentially expressed were used for Gene Ontology (GO) enrichment analysis Blast2GO software (BioBam Bioinformatics S.L., Valencia, Spain). Descriptions and GO-terms of transcript-IDs were generated from Blast2GO extracted from VectorBase. GO term enrichment analysis was performed on the significantly up-regulated genes (72% of transcripts present on microarray have GO-terms) using Blast2GO software with Fisher's exact test and false discovery rate (FDR) < 0.05 (Bariami *et al.*, 2012).

4.2.4.3 Validation of candidate genes using qRT-PCR

The best candidate genes significantly differentially expressed obtained from the microarray analysis were selected for qRT-PCR validation. The materials and method used for the qRT-PCR is described in Chapter 2. cDNA from 3 replicates of samples PG, KL, KB and NO that was used in the microarray was used for the qRT-PCR. In addition, expression of the genes was also investigated in the samples from JB that was not used in the microarray to also support their potential role in this location.

Primers used are as in Table 4.5. Primer qAAEL006727 is the primer for mutisynthetase complex (AAEL006727-RA) and primer qAAEL013623 is for trypsin (AAEL013623-RA). All samples are normalized against two housekeeping genes which are the tubulin (AAEL013229-RA) and ribosomal protein S7 (RSP7) (AAEL009496-RA) genes.

Primer Name	Sequence	Product size (base pairs)	SYBR Standards (RSq)	Efficiency (%)
qCYP6CB1 F	TGAAATCGAGCTGGATCCTT	130	0.995	90.0
qCYP6CB1 R	CTCCTAATGCTTCCATTACTCAA			
qAAEL006727 F	CTACCAGTGCGATCAAGCAG	127	0.998	91.7
qAAEL006727 R	AATCCTTTGCGTTTCACCTG			
qAAEL013623 F	TGGCCAACCTTCCTCTGTAA	123	1.000	92.4
qAAEL013623 R	CCTGCTAATTGTTGTGCTTCA			
qCYP9J26-607 F	CACGCTGCTGAAGTTTACGA	150	0.976	99.3
qCYP9J26-607 R	AAAATTTGGACAAAACCCTATTCA			
qCYP9M4 F	GGTTGATCACGAAGGACGTT	114	0.882	94.6
qCYP9M4 R	CCTGCACGAACAAATGAATG			
qCYP9J27 F	CACCGTTCAGGAGTCAGACA	128	0.995	90.0
qCYP9J27 R	TGAACATGGCACAGGTTGAT			
qCYP9J26-609 F	TGCACCACGATCCACAGTAT	109	0.997	90.7
qCYP9J26-609 R	TACCAAACGGCAGATACGC			
qTub-Aae F	CCGCACTCGAGAAGGATTAC	131	0.996	92.2
qTub-Aae R	GTGGTTCGGTTTGACTTCGT			
qRPS7-Aae F	AAGGTCGACACCTTCACGTC	131	0.986	90.1
qRPS7-Aae R	CGCGCGCTCACTTATTAGAT			

Table 4.5 Primers used for qRT-PCR for microarray candidate genes validation.

4.3 Results

4.3.1 Species Identification

The PCR based species identification (Sp. ID) method that was carried out to confirm the species of the samples showed that 100% of the samples that were previously identified by morphological differences were the correct species (Table 4.6). One of the 12 *Ae. aegypti* sample from Kuala Lumpur failed to give a result, this could be due to low DNA quality. However more F_0 samples were later obtained from Kuala Lumpur.

Aedes aegypti				
Strain	Penang	Kuala Lumpur	Johor Bharu	Kota Bharu
Sp. ID	48/48	11/12	48/48	48/48
Aedes albopictus				
Strain	Penang	Kuala Lumpur	Johor Bharu	Kota Bharu
Sp. ID	48/48	48/48	48/48	48/48

Table 4.6 Summary of the number of species correctly identified.
(X/N = number of mosquitoes correctly identified/total number of mosquitoes tested)

After conducting the PCR using the DNA extracted from the mosquitoes, gel electrophoresis was done to visualise the results. The images obtained showed clear bands indicating the correct base pair sizes for *Ae. aegypti* (bands at 120, 180 and 400 bp) and *Ae. albopictus* (major bands at 190, 200 and 290 bp) after the second PCR step which is digestion with *RsaI* enzyme (Figure 4.3 & 4.4).

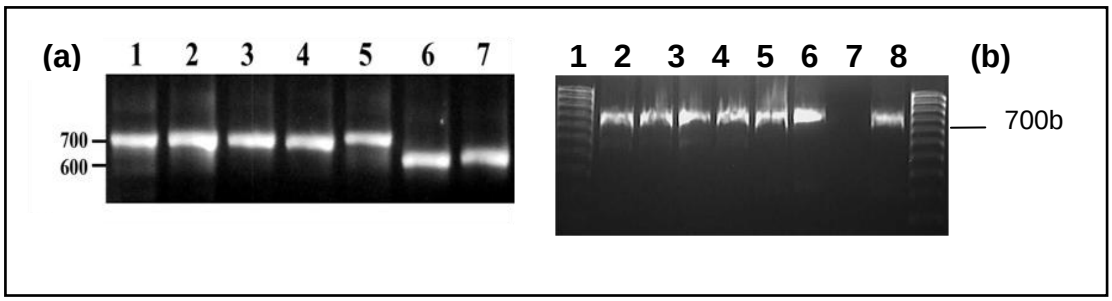


Figure 4.3 Gel electrophoresis picture showing the bands observed after conducting the PCR using internal transcribed spacer region 1 (ITS1) (step one of a two step PCR protocol) for species identification.
(a) Taken from Beebe *et al.* (2007). Lane 1: *Ae. aegypti*, Lane 2: *Ae. albopictus*, Lane 3-7: Other *Aedes* species.
(b) From the study conducted. Lane 2-5: *Ae. albopictus*, Lane 6-9: *Ae. aegypti*.

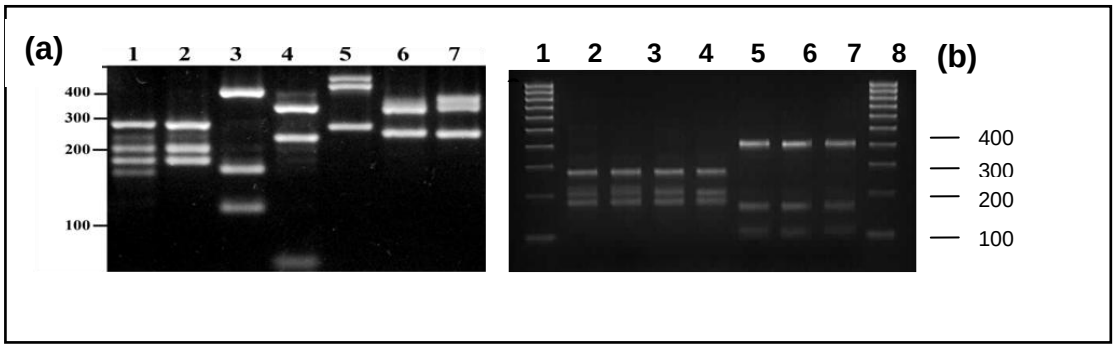


Figure 4.4 Gel electrophoresis picture showing the bands observed after digesting the PCR using *RsaI* enzyme. Restriction fragment length polymorphism (RFLP) variants (step two of a two step PCR protocol) for species identification.
(a) Taken from Beebe *et al.* (2007). Lane 1 & 2: *Ae. albopictus*, Lane 3: *Ae. aegypti*, Lane 4-7: Other *Aedes* species.
(b) From the study conducted. Lane 2-5: *Ae. albopictus*, Lane 6-8: *Ae. aegypti*.

4.3.2 *Search for potential kdr* mutations in *Ae. aegypti* in Malaysia using Pyrosequencing

4.3.2.1 Detection of *kdr* mutations associated with pyrethroids or DDT resistance

Samples from all four F_0 field strains that were genotyped by pyrosequencing for the three *kdr* mutations at codons 1011, 1016 and 1534, only showed the presence of *kdr*1534 mutation which causes a phenylalanine to cysteine substitution (F1534C).

The pyrosequencing successfully genotyped the three different genotypes expected at position 1534 as shown graphically in Figure 4.6, with peaks of

different height in the T and G region corresponding to the three possible genotypes: T/T (homozygous wild type, no mutation), G/T (heterozygous k*dr* wild type) and G/G (homozygous *kdr* wild type).

The distribution of the F1534C mutation across Malaysia is shown in Figure 4.5. Apart from the population from KL, the genotype distribution of the F1534C mutation of all other populations significantly departed from the Hardy-Weinberg equilibrium (Table 4.7). A chi-square comparison of the genotype distribution between locations revealed that the genotype distribution significantly differed between the four locations although only slightly between KB and JB (Table 4.8) which have the highest frequency for the homozygote resistant G/G genotype. Furthermore, the similarity of the F1534C frequency between KB and JB is showed by the exact frequencies of the F1534 and 1534C in both locations (Figure 4.5). Both JB and KB showed highest presence of the mutation genotype with 80%.

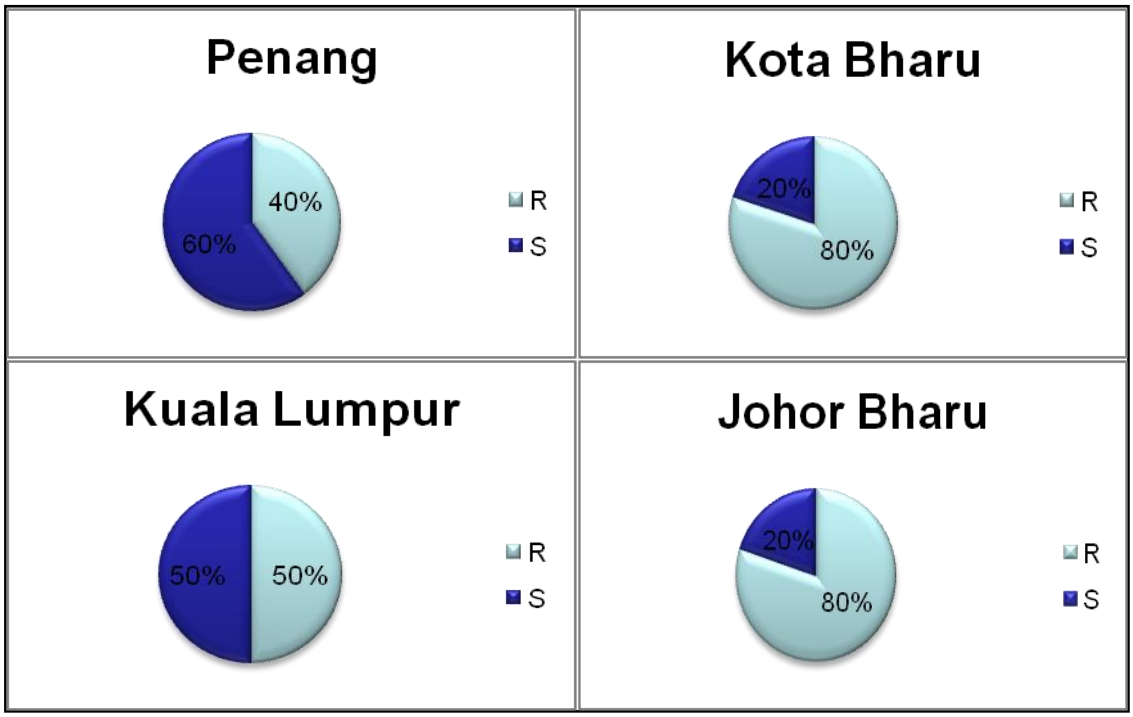


Figure 4.5 Distribution of F1534C kdr mutation (in percentage) in wild population Ae. aegypti across Malaysia.
R,S are allelic frequencies. R: samples which have resistant mutation, S: samples which does not have the mutation

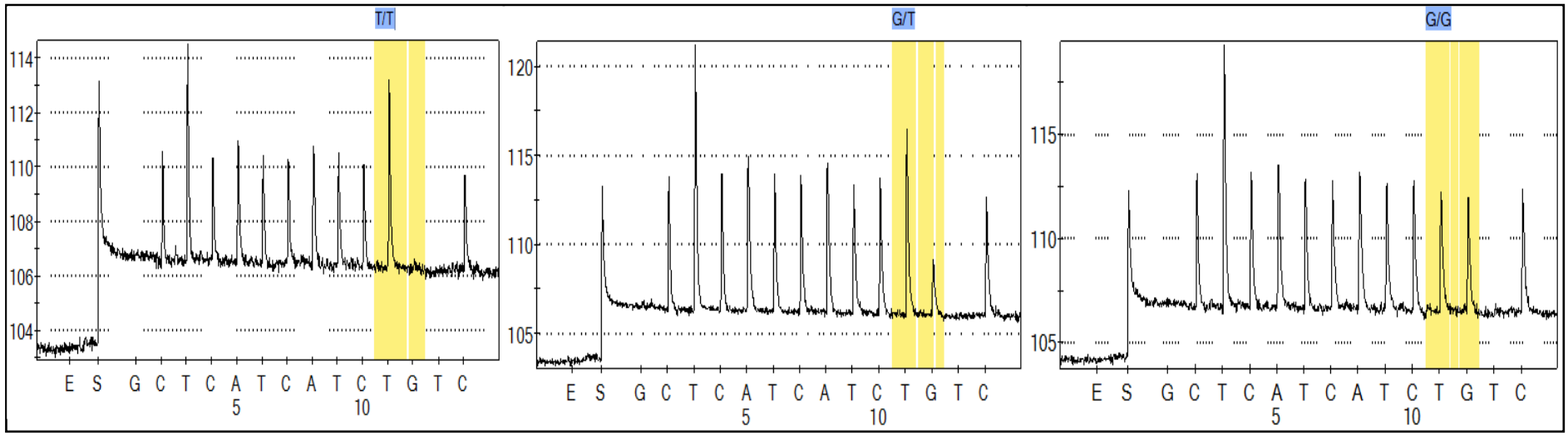


Figure 4.6 Pyrograms resulting from *kdr* F1534C pyrosequencing assay on *Aedes aegypti* mosquito samples.
SNP areas of interest are coloured yellow and peaks represent nucleotides conferring *kdr* genotype: T/T (homozygous susceptible), G/T (heterozygous), G/G (homozygous resistant).

Hardy-Weinberg equilibrium	
Population	**X^2 (p value)**
Kota Bharu	92.8 (0.000)
Kuala Lumpur	4.00 (0.135)
Penang	26.3 (0.000)
Johor Bharu	76.0 (0.000)

Table 4.7 Hardy-Weinberg equilibrium calculation for all four populations. Chi-square value (p value).

Genotype distribution			
Locations	**KL**	**PG**	**JB**
KB	101 (0.000)	132 (0.000)	9.37 (0.008)
KL	-	52.2 (0.000)	116 (0.000)
PG	-	-	433 (0.000)

Table 4.8 Comparison of genotype distribution between locations. Chi-square value (p value).

4.3.2.2 Correlation of F1534C genotypes and resistance phenotypes

The F1534C mutation was genotyped between mosquitoes alive and dead after exposure to permethrin, deltamethrin and DDT.

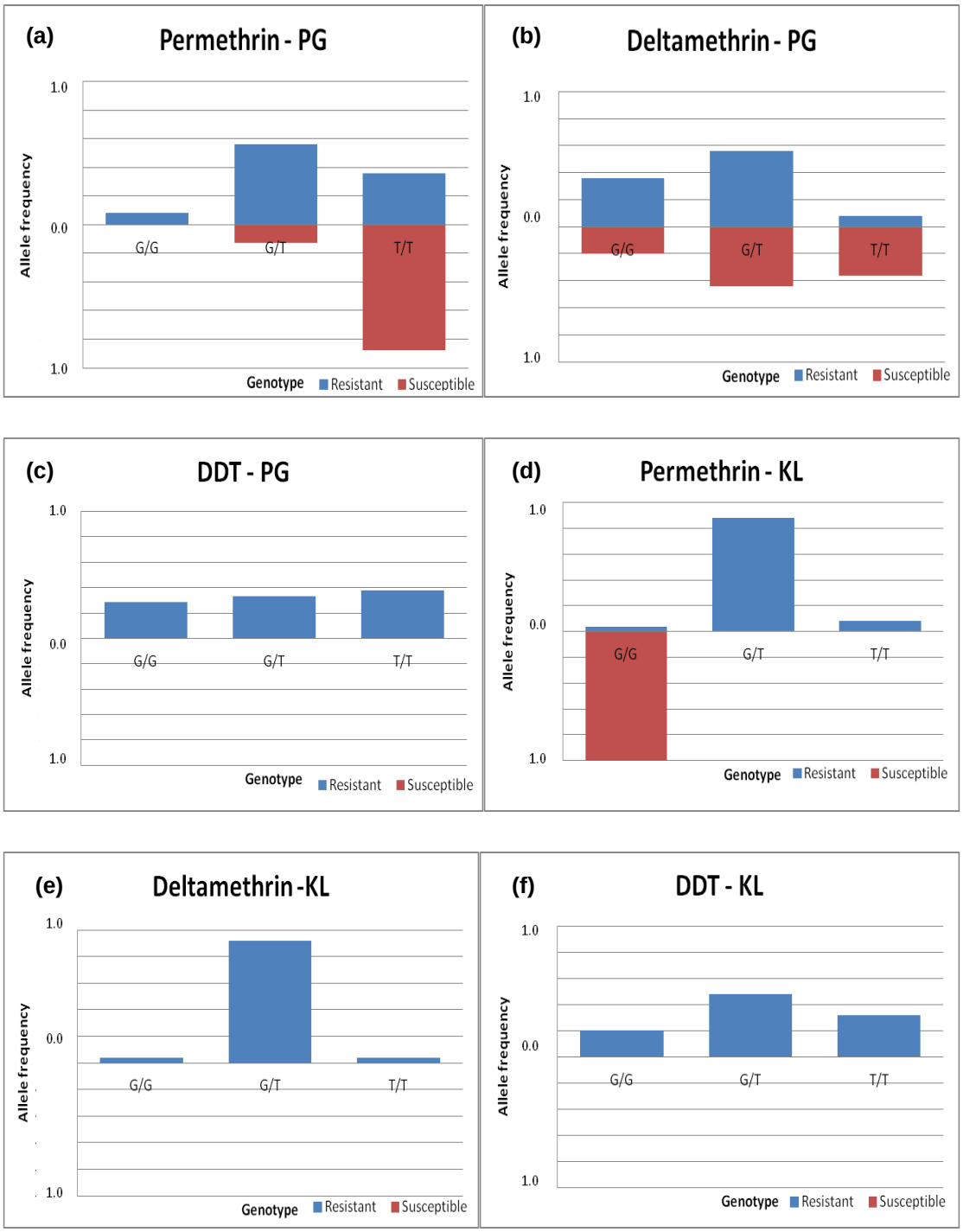
The frequency of the F1534C mutation ranges from 0.00 to 1.00 in all four populations. However, a correlation was not always found with resistance phenotype ($p< 0.05$). Odds ratio and chi square test analysis was done on the results obtained as shown in Table 4.9. However, in some samples the odds ratio and p value could not be calculated due to the low sample size for susceptible samples.

In Penang, for permethrin susceptible mosquitoes a higher frequency of T/T homozygote susceptible genotype was observed whereas the heterozygote genotype was higher in the resistant counterparts with odds ratio (OR) of 8.4375 ($p = 0.018$) (Table 4.9) (Figure 4.7 (a)) confirming a correlation between the genotype and phenotype. A similar correlation was observed for deltamethrin although with lower OR (OR = 2.455, $p = 0.027$) (Table 4.9). The OR could not be estimated for DDT exposed samples (Figure 4.7 (b) & (c)).

In Kuala Lumpur no correlation could be seen between the three genotypes and resistance phenotype since no odds ratio or p value could be calculated due to no susceptible sample obtained. However, the heterozygote genotype is higher in both of the resistant samples which could be seen in Figure 4.7 (d). This figure also shows that the F1534C homozygote resistant genotype is present in permethrin susceptible sample from Kuala Lumpur and the frequency of the heterozygote genotype is higher in the resistant samples. This could not conclude that there is a correlation between the resistant phenotype and the F1534C genotype especially since there is only a single susceptible sample and an odds ratio value could not be calculated. In figure 4.7 (e) and (f) for KL samples exposed to deltamethrin and DDT.

For samples from Johor Bharu, the frequency of the homozygote F1534C resistant genotype is equal in both susceptible and resistant samples exposed to all three insecticides (Figure 4.7 (g-h)). Permethrin exposed samples did not show a correlation between the resistant F1534C genotype and phenotypes since the OR value was <1 (OR = 0.9, p = 0.6) (Table 4.9). Deltamethrin exposed samples however, showed a correlation between the resistant genotype and phenotype with OR values of 4.65 (p = 0.001). Despite a high OR of 5 this correlation was not significant (p = 0.28) for DDT exposed samples due to the low number of susceptible mosquitoes available (Table 4.9).

Kota Bharu samples exposed to permethrin and deltamethrin showed no correlation between the F1534C resistant genotype and its' phenotype as the OR values for this sample was 0.387 (p=0.091). Surprisingly, a correlation was observed for deltamethrin but rather in the opposite way with higher frequency of the resistant allele in the dead mosquitoes (OR =0.121, p < 0.0001) (Figure 4.7 (i-l)). No correlation was also observed for DDT exposed sample with OR value of 2.66 (p=0.078) (Table 4.9).


(a)
Permethrin - PG
1.0
0.0
1.0
Allele frequency
G/G
G/T
T/T
Genotype
Resistant
Susceptible
(b)
Deltamethrin - PG
1.0
0.0
1.0
Allele frequency
G/G
G/T
T/T
Genotype
Resistant
Susceptible
(c)
DDT - PG
1.0
0.0
1.0
Allele frequency
G/G
G/T
T/T
Genotype
Resistant
Susceptible
(d)
Permethrin - KL
1.0
0.0
1.0
Allele frequency
G/G
G/T
T/T
Genotype
Resistant
Susceptible
(e)
Deltamethrin -KL
1.0
0.0
1.0
Allele frequency
G/G
G/T
T/T
Genotype
Resistant
Susceptible
(f)
DDT - KL
1.0
0.0
1.0
Allele frequency
G/G
G/T
T/T
Genotype
Resistant
Susceptible

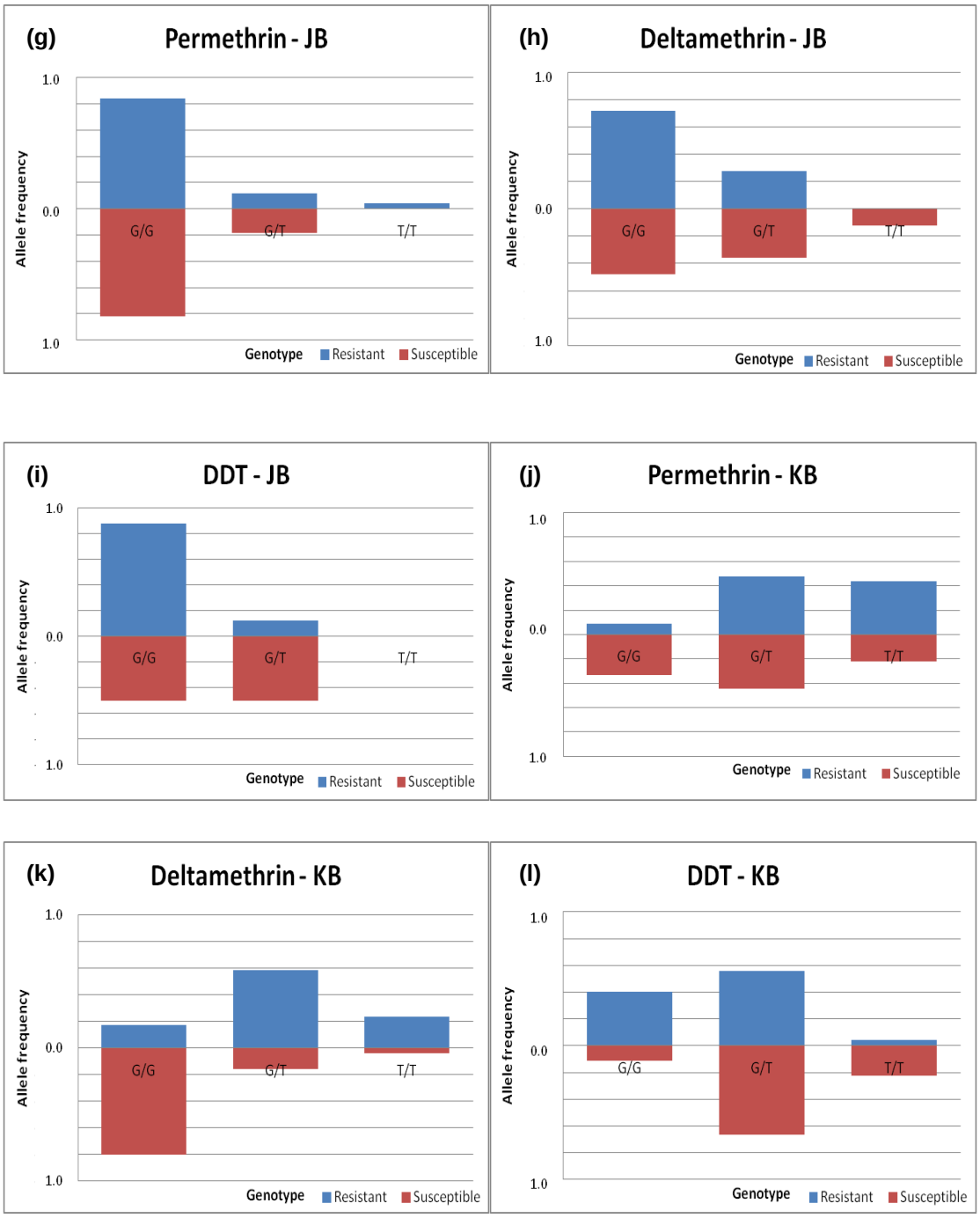


Figure 4.7 (a) – (l) Shows the genotype: phenotype correlation for the samples from Penang (PG), Kuala Lumpur (KL), Johor Bharu (JB) and Kota Bharu (KB) which was exposed to three different insecticides. X-axis: allele frequency, Y-axis: genotype.

Population	Insecticide	Phenotype	n	F1534C alleles		Odds ratio	P value
				TTC	TGC		
Penang	Perm	R	25	32	18	8.4375	0.018
		S	8	15	1		
	Delta	R	25	18	32	2.455	0.027
		S	25	29	21		
	DDT	R	25	23	19	*	*
		S	0	0	0		
Kuala Lumpur	Perm	R	25	26	24	*	*
		S	1	0	2		
	Delta	R	25	25	25	*	*
		S	0	0	0		
	DDT	R	25	28	22	*	*
		S	0	0	0		
Johor Bharu	Perm	R	25	5	45	0.900	0.600
		S	11	2	20		
	Delta	R	25	7	43	4.65	0.001
		S	25	25	33		
	DDT	R	25	3	45	5	0.280
		S	2	1	3		
Kota Bharu	Perm	R	25	31	15	0.3871	0.091
		S	9	8	10		
	Delta	R	18	18	16	0.1212	<0.0001
		S	25	6	44		
	DDT	R	25	16	34	2.6563	0.078
		S	10	10	8		

Table 4.9 Association of *kdr* F1534C allele count in all field populations with specific insecticide resistance phenotype.
n = number of samples tested. For some samples, odds ratio and p value could not be calculated.

4.3.3 Analysis of the polymorphism patterns of VGSC gene around the F1534C mutation

Due to the lack of correlation between phenotype and F1534C genotypes in most of the four field populations after conducting pyrosequencing, the polymorphism patterns of the VGSC gene was analysed around this mutation (from intron 26 to exon 29). The aim of this study was to assess a possible correlation between haplotypes of this gene and resistance phenotypes and additionally to identify trace of selection acting on this gene. 818bp fragment was therefore successfully amplified and directly sequenced to assess

haplotype correlation with resistance in all four populations between five resistant and five dead samples after permethrin exposure.

7 haplotypes were found with two major haplotypes. One was a resistant haplotype, H1-R with 49% (frequency of 30/62 from mainly resistant mosquitoes) and 30% (frequency of 19/62) for another susceptible haplotype (H2-S). Five haplotypes were singletons (only found in single samples) while one haplotype were found in six samples and another was found in five samples (Figure 4.8). The predominance of a single haplotype in resistant mosquitoes suggests that there is a correlation between polymorphism at the VGSC gene and permethrin resistance. The predominance of another haplotype in susceptible samples further supports this observation. However such correlation could originate from the presence of other *kdr* mutations than the F1534C since the signature of selection could be extensive.

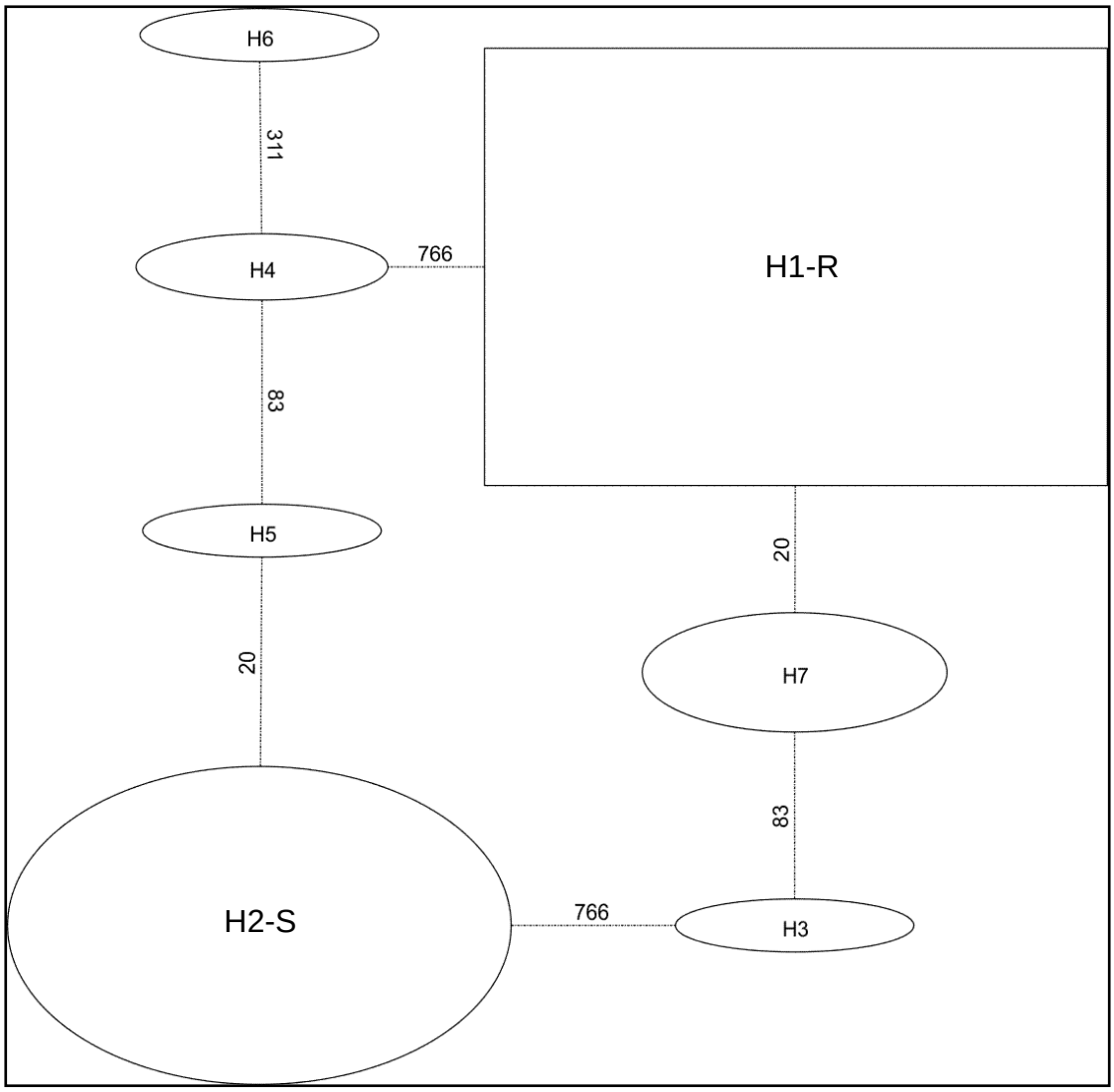


Figure 4.8 Haplotype network of F1534C mutation in the partial sequence of voltage gated sodium channel, constructed using TCS1.21 jar software.

```
          37
[      2816]
[      0316]
Hap_1  AAGG
Hap_2  GC.T
Hap_3  GC..
Hap_4  ...T
Hap_5  .C.T
Hap_6  ..AT
Hap_7  G...
```

Figure 4.9 Polymorphic amino acid positions for PCR product encompassing VGSC.

	Entire sequence region								Coding region							Non-coding region				
Samples	**N**	**S**	**h**	**Syn**	**NSyn**	**π (k)**	**D**	**D***	**S**	**h**	**Syn**	**NSyn**	**π (k)**	**D**	**D***	**S**	**h**	**π (k)**	**D**	**D***
Penang																				
Alive	10	4	3	0	2	0.00226 (1.84)	1.17	0.45	2	3	0	2	0.00169 (0.73)	0.12	-0.28	2	2	0.00294 (1.11)	1.84	1.03
Dead	12	2	3	0	0	0.00087 (0.71)	0.22	0.97	0	1	0	0	0	-	-	2	3	0.00188 (0.71)	0.22	0.97
Total	22	4	5	0	2	0.00164 (1.34)	0.62	0.14	2	3	0	2	0.00093 (0.40)	-0.6	-0.63	2	3	0.00249 (0.93)	1.61	0.85
Kuala Lumpur																				
Alive	8	3	3	0	1	0.00201 (1.64)	1.73	1.23	1	2	0	1	0.00131 (0.57)	1.44	0.89	2	2	0.00283 (1.07)	1.45	1.11
Dead	2	0	1	0	0	0	-	-	0	1	0	0	0	-	-	0	1	0	-	-
Total	10	3	3	0	1	0.00202 (1.64)	1.98*	1.15	1	2	0	1	0.00123 (0.53)	1.3	0.8	2	2	0.00294 (1.11)	1.84	1.03
Johor Bharu																				
Alive	10	3	3	0	1	0.00106 (0.87)	-0.66	-0.8	1	2	0	1	0.00046 (0.2)	-1.12	-1.24	2	3	0.00176 (0.67)	-0.18	-0.28
Dead	10	1	2	0	0	0.00065 (0.53)	1.3	0.8	0	1	0	0	0	-	-	1	2	0.00141 (0.53)	1.3	0.8
Total	20	3	3	0	1	0.00083 (0.68)	-0.53	-1.25	1	2	0	1	0.00023 (0.1)	-1.16	-1.54	2	3	0.00153 (0.58)	0.06	-0.59
Kota Bharu																				
Alive																				
Dead	10	3	2	0	1	0.00131 (1.07)	0.02	1.15	1	2	0	1	0.00082 (0.36)	0.01	0.8	2	2	0.00188 (0.71)	0.02	1.03
Total																				
Combined																				
Alive	28	4	5	0	2	0.00193 (1.58)	1.4	0.52	2	3	0	2	0.00130 (0.57)	0.21	-0.71	2	3	0.00267 (1.01)	2.02*	0.82
Dead	34	3	5	0	1	0.00181 (1.48)	2.31*	0.93	1	2	0	1	0.00115 (-0.50)	1.56	0.58	2	4	0.0026 (0.98)	1.97	0.79
Total	62	4	7	0	2	0.00184 (1.50)	1.65	-0.15	2	3	0	2	0.00120 (0.52)	0.38	-0.95	2	4			

Table 4.10 Polymorphism parameters of the VGSC fragment between permethrin resistant and susceptible *Ae. aegypti* across Malaysia.
N= number of sequences (2n); S, number of polymorphic sites; h, Number of haplotypes; (haplotype diversity); Syn, synonymous; Nsyn, non-synonymous; π, nucleotide diversity (k= mean number of nucleotide differences); Tajima's D and Fu and Li's D* statistics.

4.3.4 Genotyping of other *kdr* mutations and the analysis of the polymorphisms of VGSC

From the genotyping of F1534C mutation and the analysis of the polymorphisms in VGSC, a strong or significant correlation between the resistant F1534C genotype and phenotype could not be found in most of the samples tested. However, there were some positive correlation for permethrin in Penang, deltamethrin and DDT in Johor Bharu and DDT resistance in Kota Bharu. Also from haplotype network, there is a haplotype predominant in resistant and another in susceptible which could be sign of correlation.

In an effort to detect potential additional *kdr* mutations in *Ae. aegypti* populations in Malaysia, the cDNA fragment spanning exon 19 to 31 was successfully amplified and sequenced. The PCR amplification successfully generated 2586 bp of PCR product. The amplification of exon 19 to 31 of cDNA known to harbour potential *kdr* mutations detected the V1016G mutation in PG, KL and KB samples (Figure 4.10). These samples showed a change in the nucleotide sequences from a susceptible GTA to GGA or having both G and T nucleotides (heterozygous for the K mutation) (Figure 4.10 & 4.11).

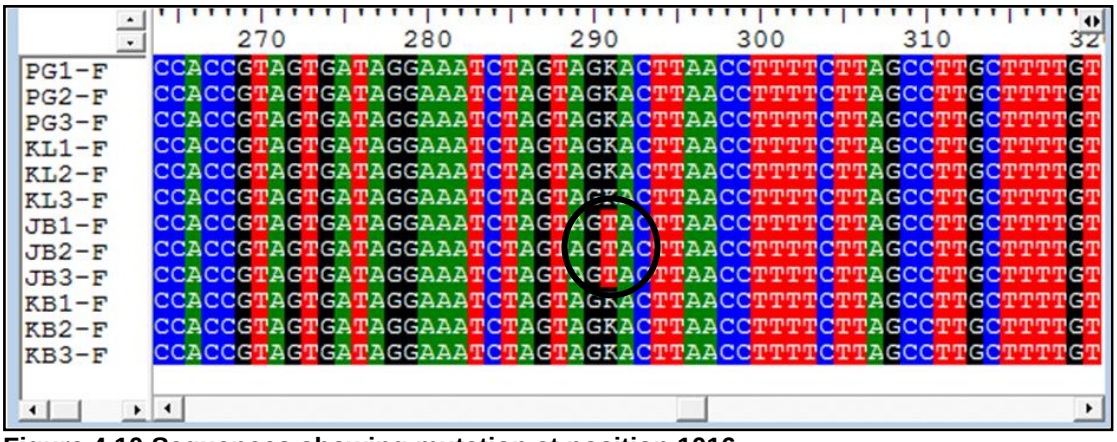


Figure 4.10 Sequences showing mutation at position 1016.
Nucleotide sequence at position 291 shows the mutation in all the samples tested. Sequences of samples from Johor Bharu are susceptible (circled).

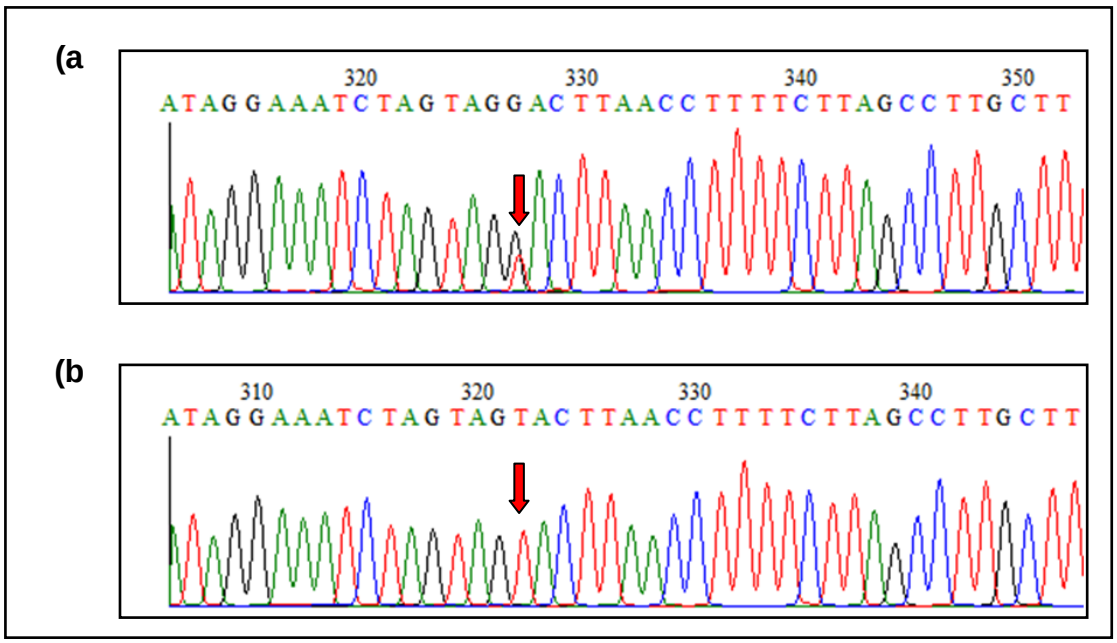


Figure 4.11 Chromatograph of sequences showing mutation at position 1016 . Example of samples (a) Kuala Lumpur, (b) Johor Bharu.
Red arrow marks the peak of mutation.

4.3.5 Genotyping of *kdr* mutation using allele specific PCR

Through direct sequencing of exon 19 to 31 of the VGSC, the presence of a T to G substitution in exon 21 resulting in *kdr* V1016G mutation was detected in the *Ae. aegypti* samples from Malaysia (control samples, non-exposed to insecticide). Since the V1016G mutation was undetected using the pyrosequencing method, another method described by (Saavedra-Rodriguez *et al.*, 2007) was used. The PCR based assay could be read through agarose gel or as a melting curve. Our study used the melting curve detection method.

Preliminary test was conducted on five F_0 samples from each of the four field populations to detect the presence of both mutations at the 1016 codon (V1016I and V1016G). Only V1016G was detected in all samples. An example of the result for the melt curve patters are from the MxPro qPCR software (Agilent technologies, USA) are shown in Figure 4.12.

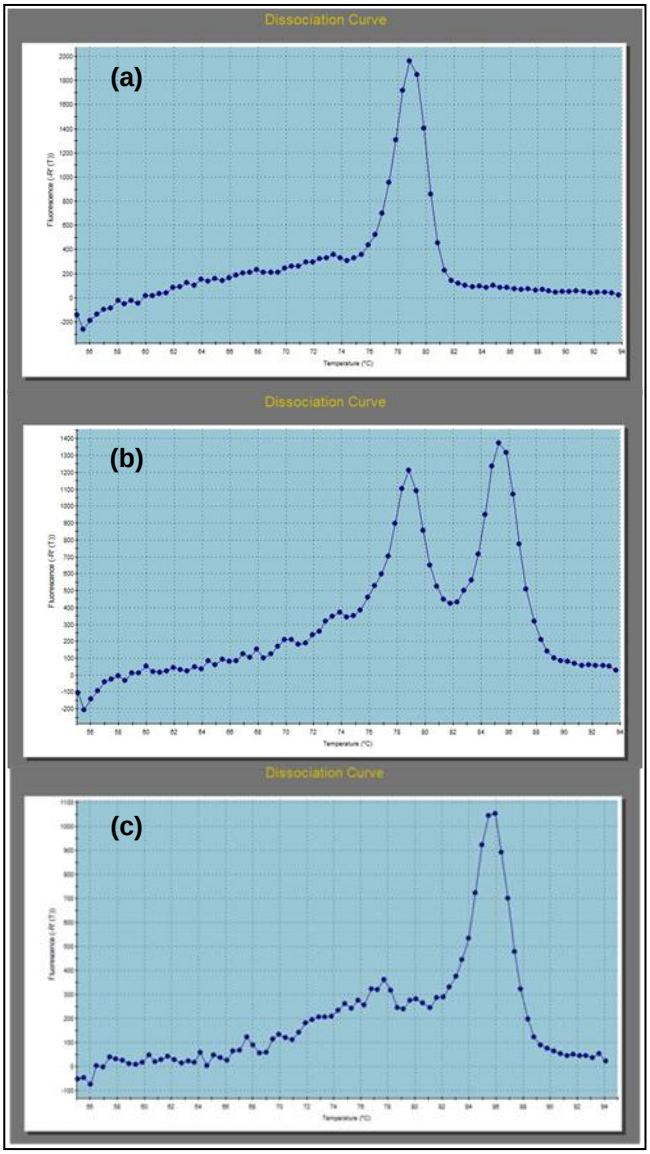


Figure 4.12 Example of melt curve patterns for V1016G genotype.
(a) Homozygous susceptible (b) Heterozygous resistant (c) Homozygous resistant

The assay was conducted on 48 F_0 mosquitoes from each location and the distribution of the V1016G mutation across Malaysia is shown in Figure 4.13. Field samples from PG showed the highest percentage for the resistant allele frequency with 39% followed by KL with 36% and JB with 22%. KB showed the lowest presence of the mutation allele with 20%.

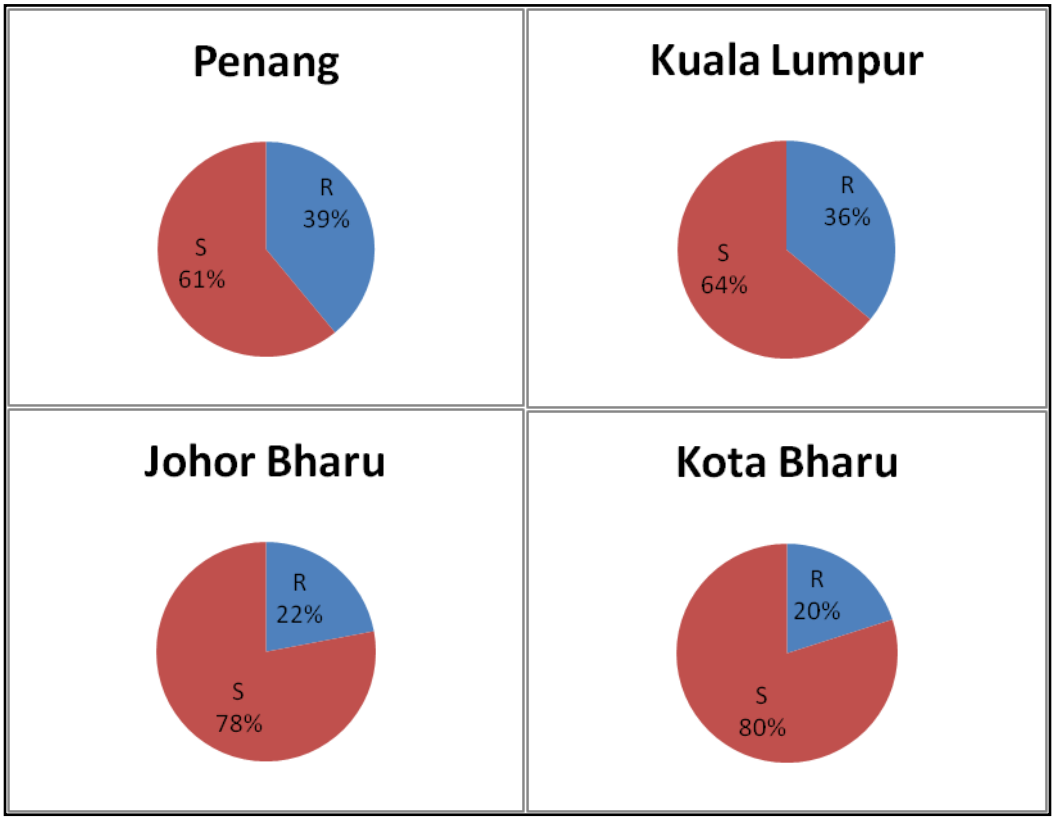


Figure 4.13 Distribution of V1016G *kdr* mutation (in percentage) in wild population *Ae. aegypti* across Malaysia.
R: samples which have resistant mutation, S: samples which does not have the mutation

Samples from the F_2 generation dead and alive that were exposed to permethrin, deltamethrin and DDT from all four strains (Table 4.2) were also genotyped to assess correlation between the V1016G and resistance phenotype. As in the *kdr* F1534C mutation, the frequency for V1016G ranges from 0.00 to 1.00 in all four populations. No significant correlation was observed between V1016G and resistance phenotypes for all 3 insecticides with low OR and P>0.05 (Table 4.11) for most of the samples except for permethrin and DDT exposed samples from Johor Bharu. In some samples the odds ratio and p value could not be calculated due to the low sample size.

In Johor Bharu, a correlation was observed for permethrin and DDT exposed samples but in the opposite way with higher frequency of the susceptible allele in the alive mosquitoes with OR values of 0.011 ($p < 0.0001$) and 0.014 ($p < 0.0001$) respectively (Table 4.11).

Population	Insecticide	Phenotype	n	V1016G alleles		Odds ratio	P value
				GTA	GGA		
Penang	Perm	R	25	25	25	1.2857	0.6629
		S	8	9	7		
	Delta	R	25	28	22	1.1786	0.6892
		S	25	30	20		
	DDT	R	25	28	22	*	*
		S	0	0	0		
Kuala Lumpur	Perm	R	25	20	30	*	*
		S	1	2	0		
	Delta	R	25	24	26	*	*
		S	0	0	0		
	DDT	R	25	22	28	*	*
		S	0	0	0		
Johor Bharu	Perm	R	25	45	5	0.0111	<0.0001
		S	11	2	20		
	Delta	R	25	42	8	1.1156	0.8414
		S	24	41	7		
	DDT	R	25	48	2	0.0139	0.0016
		S	2	1	3		
Kota Bharu	Perm	R	25	42	8	0.3537	0.0794
		S	10	13	7		
	Delta	R	18	23	13	2.004	0.1502
		S	25	39	11		
	DDT	R	25	39	11	0.3447	0.0544
		S	10	11	9		

Table 4.11 Association of *kdr* V1016G allele count in all field populations with specific insecticide resistance phenotype.
n = number of samples tested. For some samples, odds ratio and p value could not be calculated.

4.3.6 Sequencing of *Ace-1* gene

PCR amplification conducted on the *Ace-1* gene successfully generated a PCR product. The gene was amplified using forward and reverse primers which produced a PCR product of 2562 bp as expected for all the bendiocarb resistant cDNA samples (Figure 4.14).

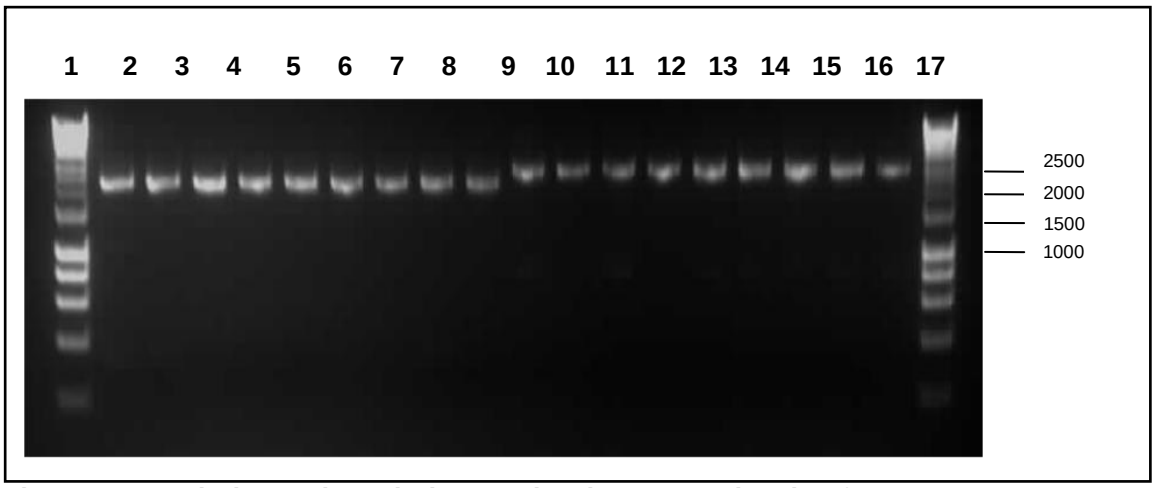


Figure 4.14 Gel electrophoresis image showing strong bands after *Ace-1* gene PCR amplification.
Lane 2-10: *Ae. albopictus*, Lane 11-19: *Ae. aegypti*

Unfortunately, direct sequencing of the *Ace-1* gene was uninformative since the presence of mutations could not be determined due to overlapping peaks. The sequencing data obtained was not of good quality in both forward and reverse directions. This was due to the presence of alternative splicing as pooled of cDNA were sequenced. Sequence chromatograms exhibited overlapping peaks for most of the sequence (Figure 4.15). There were double peaks starting at nucleotide position 500 for the forward sequence and 600 for the reverse sequence preventing the detection of polymorphisms. The best alternative to obtain precise results is to clone the samples but unfortunately time was a constraint when conducting this research.

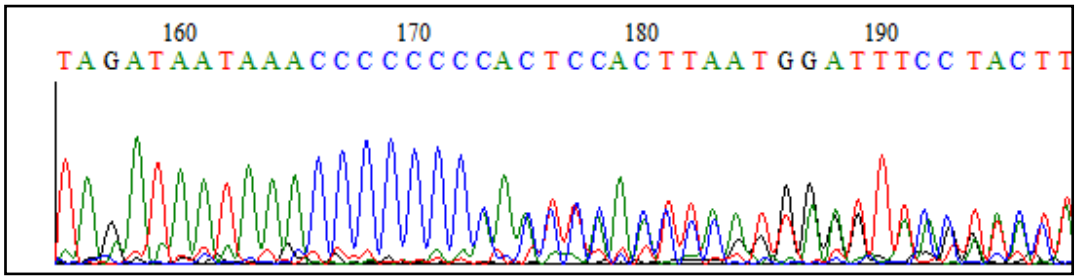


Figure 4.15 Nucleotide sequence chromatograms from Bioedit software showing the presence of alternative splicing in a sample from Kuala Lumpur.

4.3.7 Genome-wide transcriptome profiling of *Ae. aegypti* populations in Malaysia using microarray

The microarrays were used to perform a genome-wide transcription analysis between the susceptible New Orleans laboratory strain and non-exposed field strains (control) from Penang (PG), Kuala Lumpur (KL) and Kota Bharu (KB). The experimental design of the microarray is as described in Section 4.2.4.1.

Before hybridisation of the cRNA samples, they were first labelled and their quality was assessed. All 12 RNA pools (3 susceptible (S) and 9 control field samples (C)) of 10 female mosquitoes were successfully labelled with both Cy3 and Cy5 dyes using the Quick Amp Labelling Kit (Agilent, USA). Bioanalyzer analysis after labelling confirmed the quality of cRNA with large peaks between 100-2000 nucleotides recorded in all samples (example of traces in (Figure 4.16). All the samples were used for microarray hybridisation.

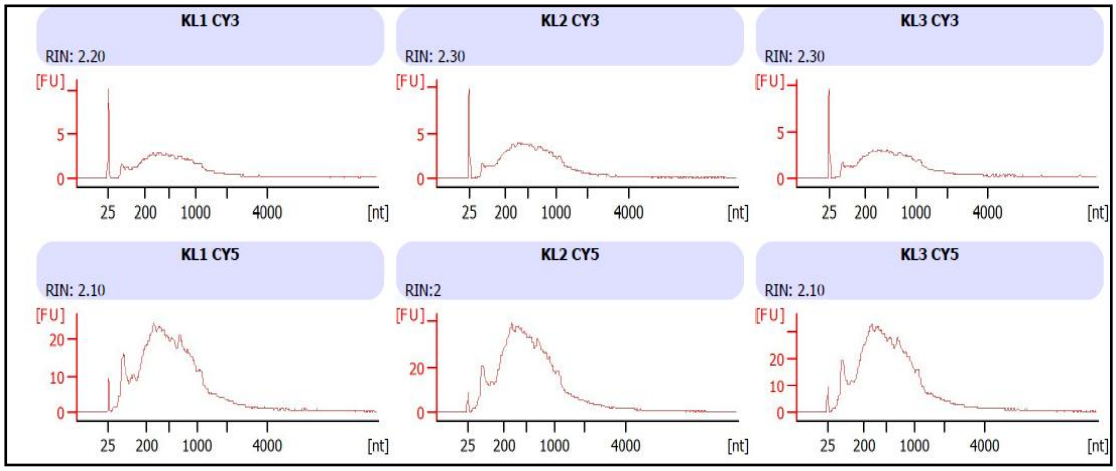


Figure 4.16 example of Bioanalyzer traces from Cy3 and Cy5 labelled cRNA samples from Kuala Lumpur.

The data from all the arrays conducted showed good quality with the overall QC score of 11 out of 11. The data was then analysed. The selection criterion for differentially expressed genes was that the transcript detected was > 2 fold in any direction, with a p-value of 0.01 after Benjamin and Bonferroni correction.

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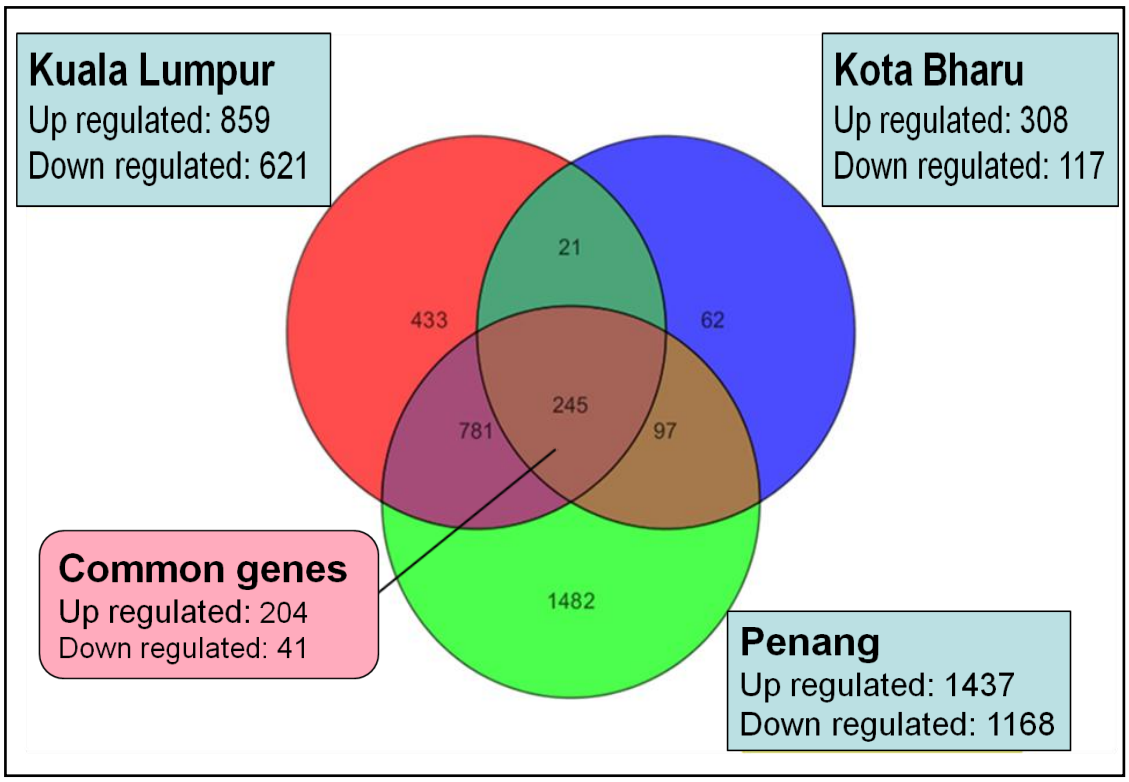

Figure 4.17 Venn diagram of differentially transcribed genes of *Aedes aegypti* from microarray data. (p= 0.01)

The number of differentially transcribed genes after analysis could be seen in the Venn diagram (Figure 4.17). Penang had the most number of differentially transcribed probes with 2605 gene probes, followed by Kuala Lumpur with 1480 gene probes and Kota Bharu with 425 gene probes. The number of commonly up-regulated probes in all population is 204 and 41 probes were down-regulated.

Genes commonly up-regulated in the three locations

Data from the microarray were analysed to select potential resistance candidate genes. The commonly up-regulated gene probes were assessed since the likely involvement of those genes in the resistance across these locations is higher. The 204 up-regulated gene probes consisted of various gene families such as protein synthesis, ion transport, detoxification and others (Table 4.12). The most commonly over-expressed gene was the anionic-trypsin which is found in the midgut of mosquitoes to hydrolyse proteins after blood meals.

Out of the commonly up-regulated detoxification genes, cytochrome P450 genes were the dominant genes found with 9 genes observed, followed by and 1 carboxylesterase. CYP6CB1 gene was the most over-expressed gene with the highest FC value in PG (212.00) followed by Kuala Lumpur and Kota Bharu with FC values of 124.70 and 36.40 respectively. Another detoxification gene that was over-expressed was the CYP9J26 gene (AAEL014609-RA) with a similar expression level in PG and KL with 7.20 and 7.70 FC values. The expression was lower for this gene in KB with FC value of 4.40. CYP9M4 and CYP9J27 had the similar expression level in the three locations. In KL the FC value for CYP9M4 and CYP9J27 was 13.20 and 13.80, followed by PG with 7.20 and 7.40 and KB with 4.10 and 3.70. Another cytochrome P450 which was over-expressed in all the locations was the transcript AAEL014614-RA which had the closest hit to CYP9J4 in *An. gambiae* after NCBI BLAST. The FC value for this gene was highest in KL with 13.80 followed by PG and KB with 2.90 and 2.10 FC values respectively. The unique carboxylesterase gene which was commonly up-regulated in the three locations had similar low FC values for all the locations ranging from 2.00 to 2.80 (Table 4.12).

Genes commonly up-regulated in the two locations

From the list of probes over-expressed only in between KL and PG but not in KB, eight genes linked to detoxification and resistance can be observed. Another transcript of CYP9J26 (AAEL014607-RA) was the most over-expressed cytochrome P450 with FC values more than 350 in both locations which was by far more than the FC values for the same gene but with a different transcript (AAEL014609-RA) in the commonly over-expressed genes for all three locations. Similarly, a different transcript of the CYP9J27 (AAEL014606-RA) was also up-regulated in KL (FC value 16.90) and PG (FC value 12.20) only. Other cytochrome P450s included CYP4C52, CYP6AH1 and a transcript with the closest hit to CYP6P4 in *An. gambiae*, a cytochrome P450 associated with permethrin resistance in *An. gambiae* (Tene *et al.*, 2013) and *An. arabiensis* (Witzig *et al.*, 2013). Also, GSTE2 was commonly

up-regulated in KL and PG with FC values of 4.50 and 2.40 respectively (Table 4.13). This gene has been reported to be responsible for DDT resistance in Malaria vectors (David et al., 2005, Djouaka et al., 2011) and *Ae. aegypti* (Lumjuan *et al.*, 2007) (Table 4.14).

For commonly over-expressed genes in KB and PG, only two genes could be observed. One cytochrome P450 CYP6BB2 with FC value of 2.00 in KB and 5.20 in PG was recorded. Another gene up-regulated was the cytochrome b561 which has been previously found in other insecticide resistance study in *Ae. aegypti* (Bariami *et al.*, 2012) (Table 4.14).

Only a cytochrome P450 with a transcript with the closest hit to CYP9M1 in *An. gambiae* was commonly up-regulated in KL and KB. The FC values were low with 2.50 in KB and 2.40 in KL (Table 4.14).

Genes up-regulated only in a single location

When observing the list of up-regulated genes in a single location only, PG had the most number of up-regulated detoxification genes with a predominance of cytochrome P450 among which was the CYP6P12 (FC value 6.50) an ortholog of CYP6P4 in *An. gambiae*. Other cytochrome P450 genes are the CYP6BB2 (a different probe than in the commonly up-regulated in PG and KB), CYP6N6, CYP325X1, CYP4D37, CYP9J10, CYP6Z8 and others. Other genes that could be found belong to GST (GSTS1), carboxylesterase (transcript AAEL000905-RA and CCEAE1B) and ABC transporters (Table 4.14).

In KL only, mainly cytochrome P450 could be observed among which was the CYP9J28 gene with the highest FC value of 10.80 and had been previously reported to confer pyrethroid resistance (Stevenson *et al.*, 2012). Other cytochrome P450s were CYP18A1 and CYP9M6 (Table 4.14).

The genes up-regulated in KB only consisted of three genes which were a cytochrome P450 (CYP9M7), an ABC transporter and a UDP glycuronosyl transferase (Table 4.14).

Genes commonly down-regulated in the three locations

Out of the top genes commonly down-regulated in PG, KL and KB the highest down-regulated was the domain-containing protein cg6693. Glutamine synthetase seemed to be consistently down-regulated with two probes being consistently at the top. One detoxification gene could be observed in the commonly down-regulated gene which was the cytochrome P450 CYP325M2 (Table 4.13 & 4.15).

Table 4.12 Top 50 commonly up-regulated probes in *Ae. aegypti* in all three locations in comparison with susceptible New Orleans strain. FC = fold change. (p = 0.01)

Probe Name	Systematic Name	Blast2GO Annotation	Kota Bharu vs NO		Kuala Lumpur vs NO		Penang vs NO	
			Absolute FC	Corrected p-value	Absolute FC	Corrected p-value	Absolute FC	Corrected p-value
CUST_920_PI424980000	AAEL013623-RA	anionic trypsin-2	43.86	0.044294	759.15	0.00108	581.43	2.67E-04
CUST_3568_PI424980000	AAEL011733-RA	transcription elongation regulator 1	56.06	0.036507	448.21	0.001285	255.18	3.55E-04
CUST_6004_PI424980000	AAEL009891-RA	isoform a	136.08	0.001126	299.55	0.003494	167.27	0.001721
CUST_8758_PI424980000	AAEL000183-RA	hypothetical protein	148.20	1.49E-04	269.77	0.001346	149.85	4.07E-04
CUST_11373_PI424980000	AAEL000888-RA	single stranded dna binding protein	153.09	2.29E-04	232.52	0.001143	100.23	9.22E-04
CUST_11374_PI424980000	AAEL000888-RB	single stranded dna binding protein	129.32	2.21E-04	210.12	1.65E-04	136.52	7.94E-04
CUST_3849_PI424980000	AAEL001806-RA	microsomal triglyceride transfer protein large subunit	15.54	0.028302	177.68	0.003888	138.31	4.28E-04
CUST_2177_PI424980000	AAEL013693-RA	excision repair cross-complementing 1 ercc1	34.69	0.047021	163.73	0.001663	193.23	3.46E-04
CUST_9198_PI424980000	AAEL006727-RA	multisynthetase auxiliary	238.54	3.45E-04	159.16	0.002556	185.03	1.73E-04
CUST_9199_PI424980000	AAEL006727-RB	multisynthetase auxiliary	296.08	2.21E-04	148.03	0.002085	298.80	2.67E-04
CUST_2020_PI424980000	AAEL012726-RA	hypothetical protein	148.57	2.21E-04	137.98	0.001687	98.50	3.26E-04
CUST_2845_PI424980000	AAEL009018-RA	cytochrome p450 (CYP6CB1)	36.37	0.048806	124.69	0.001285	211.97	7.56E-04
CUST_10987_PI424980000	AAEL003672-RA	zinc finger protein	40.78	0.003836	112.54	0.001635	126.80	2.69E-04
CUST_5231_PI424980000	AAEL011383-RA	mage protein	28.97	0.04977	108.64	0.005995	106.99	3.90E-04
CUST_2768_PI424980000	AAEL010097-RA	exuperantia 2	17.00	0.04184	99.63	0.001143	109.67	1.40E-04
CUST_2609_PI424980000	AAEL015136-RA	niemann-pick type c-	55.13	3.45E-04	97.61	0.001467	173.74	1.40E-04
CUST_1955_PI424980000	AAEL008525-RA	zinc finger imprinted 3	61.41	8.08E-04	91.20	0.001346	20.60	0.005476
CUST_6355_PI424980000	AAEL003679-RA	zinc finger protein	28.29	7.33E-04	77.60	0.002124	42.45	8.56E-04
CUST_11261_PI424980000	AAEL004545-RA	cas1 domain-containing protein 1-like	16.11	0.02661	66.05	0.001053	70.17	5.16E-04
CUST_7094_PI424980000	AAEL012673-RA	ubiquitin conjugating enzyme	24.00	0.004167	65.55	0.003194	45.66	0.001165

		7 interacting protein						
CUST_3521_PI424980000	AAEL010555-RA	sterol regulatory element-binding protein 1	55.42	2.94E-04	65.14	0.003143	78.23	8.14E-04
CUST_2969_PI424980000	AAEL000321-RA	acetyl-coa synthetase	25.12	9.29E-04	54.66	0.002708	29.79	0.004955
CUST_5474_PI424980000	AAEL007632-RA	myosin light chain kinase	8.37	0.008264	54.57	0.002445	35.71	0.001564
CUST_11672_PI424980000	AAEL010222-RB	gata transcription factor	29.35	3.07E-04	47.12	0.002157	18.17	7.42E-04
CUST_13241_PI424980000	AAEL012487-RA	hypothetical protein	9.45	0.037488	46.50	0.002874	22.19	0.006043
CUST_12609_PI424980000	AAEL005786-RA	isoform a	16.33	2.94E-04	45.41	0.005995	20.72	7.51E-04
CUST_2242_PI424980000	AAEL003455-RA	isoform a	16.12	0.047723	42.08	0.00754	64.79	9.22E-04
CUST_13231_PI424980000	AAEL002446-RA	isoform b	16.05	0.034975	40.15	0.003882	29.95	3.69E-04
CUST_13389_PI424980000	AAEL012357-RB	hemolymph protein	11.48	0.048806	38.26	0.003208	69.58	0.002092
CUST_7459_PI424980000	AAEL013110-RA	isoform a	17.08	0.002495	37.16	0.003682	14.45	0.002239
CUST_12920_PI424980000	AAEL003700-RA	zinc finger protein	17.07	9.29E-04	36.87	0.002139	43.03	6.70E-04
CUST_6811_PI424980000	AAEL014583-RA	60s acidic ribosomal protein p2	23.36	0.002899	34.51	0.00108	38.49	1.44E-04
CUST_1318_PI424980000	AAEL005745-RA	gpcr class a orphan receptor 18 (agap005002-pb)	19.49	5.38E-04	33.65	0.003315	14.15	0.003402
CUST_6812_PI424980000	AAEL014583-RB	60s acidic ribosomal protein p2	22.10	0.008264	33.58	0.00108	40.38	1.34E-04
CUST_11048_PI424980000	AAEL010247-RA	cg5913 cg5913-pa	14.88	0.002906	33.27	0.00108	37.73	7.03E-04
CUST_11671_PI424980000	AAEL010222-RA	gata transcription factor	26.73	3.45E-04	32.81	0.001309	13.35	6.29E-04
CUST_658_PI424980000	AAEL012357-RA	hemolymph protein	14.37	0.047514	32.40	0.005494	49.00	7.69E-04
CUST_4722_PI424980000	AAEL004530-RA	zinc finger protein	12.69	0.030913	31.97	0.003494	41.14	3.26E-04
CUST_7952_PI424980000	AAEL009828-RA	vacuolar fusion protein ccz1 homolog	6.58	0.017094	31.97	0.003045	9.94	0.001053
CUST_6813_PI424980000	AAEL014583-RC	60s acidic ribosomal protein p2	21.47	0.002906	30.30	0.001309	38.47	1.04E-04
CUST_2975_PI424980000	AAEL009076-RA	nadh dehydrogenase subunit 4	13.26	0.011725	28.77	0.004492	35.40	3.07E-04
CUST_5654_PI424980000	AAEL012983-RA	king isoform b	13.54	0.022381	28.57	0.003875	28.33	8.37E-04

CUST_7348_PI424980000	AAEL000229-RA	sialokinin i preproprotein	75.55	8.33E-04	28.20	0.022194	114.84	3.10E-04
CUST_6523_PI424980000	AAEL007147-RA	hypothetical protein	7.44	0.026524	27.16	0.00593	18.52	6.50E-04
CUST_11049_PI424980000	AAEL010247-RB	family with sequence similarity member b-like	11.32	0.002906	25.63	0.002139	21.68	0.001045
CUST_3982_PI424980000	AAEL004187-RA	gpcr class a orphan receptor 18 (agap005002-pa)	15.42	7.72E-04	24.97	0.001687	8.45	0.011122
CUST_11072_PI424980000	AAEL007984-RA	hypothetical protein	8.06	0.02135	24.45	0.006746	14.29	0.002093
CUST_9292_PI424980000	AAEL003349-RA	nadph-cytochrome p450 reductase	6.63	0.044205	24.23	0.004212	8.85	1.22E-04
CUST_6584_PI424980000	AAEL013215-RA	sulfonylurea receptor abc transporter	10.65	0.002906	24.01	0.00503	4.57	0.009755
CUST_13373_PI424980000	AAEL003052-RA	isoform a	5.81	0.0105	22.96	0.004212	5.88	0.002497

Table 4.13 Top 20 commonly down-regulated probes in *Ae. aegypti* in all three locations in comparison with susceptible New Orleans strain. FC = fold change. (p = 0.01)

Probe Name	Systematic Name	Blast2GO Annotation	Kota Bharu vs NO		Kuala Lumpur vs NO		Penang vs NO	
			Absolute FC	Corrected p-value	Absolute FC	Corrected p-value	Absolute FC	Corrected p-value
CUST_3200_PI424980000	AAEL013569-RA	domain-containing protein cg6693	31.21	0.02558	33.07	0.046552	53.72	2.35E-04
CUST_12358_PI424980000	AAEL001887-RB	glutamine synthetase 2	4.02	0.002906	18.63	0.019508	12.71	0.001995
CUST_12357_PI424980000	AAEL001887-RA	glutamine synthetase 2	3.79	4.27E-04	13.70	0.025112	16.18	0.001403
CUST_7707_PI424980000	AAEL000535-RA	galactose-specific c-type	7.07	0.019551	12.85	0.003315	12.90	2.13E-04
CUST_3015_PI424980000	AAEL004397-RA	ankyrin repeat	2.70	1.68E-04	11.64	0.046495	31.81	0.001583
CUST_11275_PI424980000	AAEL001828-RA	hypothetical protein	2.52	0.009108	9.30	0.037338	16.73	6.28E-04
CUST_4719_PI424980000	AAEL010221-RA	gata transcription factor gatad	3.38	0.029791	7.02	0.012952	16.43	0.001281
CUST_1302_PI424980000	AAEL007347-RA	serine protease	2.35	0.002536	5.84	0.017178	5.67	0.005945
CUST_3177_PI424980000	AAEL001274-RA	hypothetical protein	5.42	0.004642	5.79	0.003556	8.13	3.69E-04
CUST_5528_PI424980000	AAEL006280-RA	juvenile hormone acid methyltransferase	2.05	0.00194	5.56	0.026461	5.28	0.00248
CUST_5946_PI424980000	AAEL008224-RA	hypothetical protein	2.17	0.044052	5.53	0.011167	26.36	0.001065
CUST_22_PI424980000	AAEL012769-RA	cytochrome p450 (CYP325M2)	2.95	0.006449	5.36	0.011548	6.39	9.43E-04
CUST_11250_PI424980000	AAEL004342-RA	odorant-binding protein	3.68	0.019184	5.11	0.038264	13.82	4.95E-04
CUST_2153_PI424980000	AAEL015650-RA	isoform a	3.37	0.009584	4.97	0.009499	7.92	2.69E-04
CUST_8546_PI424980000	AAEL012850-RA	isoform a	3.50	0.012497	4.86	0.019799	8.60	2.67E-04
CUST_1674_PI424980000	AAEL015468-RA	hypothetical protein	2.61	0.021134	4.79	0.008665	3.66	0.00596
CUST_10633_PI424980000	AAEL002447-RA	hypothetical protein	2.12	0.013594	4.67	0.011003	3.11	7.97E-04
CUST_7240_PI424980000	AAEL014163-RA	serine protease	2.51	0.017204	4.64	0.0141	4.88	0.003521
CUST_6420_PI424980000	AAEL006466-RA	chondroitin synthase	2.40	0.013921	4.51	0.003494	71.29	9.62E-04
CUST_2695_PI424980000	AAEL009825-RA	60s ribosomal protein l13a	2.12	0.002906	4.14	0.013701	18.05	3.02E-04

Table 4.14 Probes from detoxification genes & genes linked with resistance commonly up-regulated in *Ae. aegypti* in all three locations in comparison with susceptible New Orleans strain. FC = fold change. (p = 0.01)

Probe Name	Systematic Name	Blast2GO Annotation	Kota Bharu vs NO		Kuala Lumpur vs NO		Penang vs NO	
			Absolute FC	Corrected p-value	Absolute FC	Corrected p-value	Absolute FC	Corrected p-value
Common to all three locations								
CUST_2845_PI424980000	AAEL009018-RA	cytochrome p450 (CYP6CB1)	36.40	0.04881	124.70	0.00128	212.00	0.00076
CUST_9292_PI424980000	AAEL003349-RA	nadph-cytochrome p450 reductase	6.60	0.0442	24.20	0.00421	8.90	0.00012
CUST_12457_PI424980000	AAEL014689-RA	nadph cytochrome p450	5.30	0.0303	15.10	0.00163	11.00	0.00022
CUST_105_PI424980000	AAEL014609-RA	cytochrome p450 (CYP9J26)	4.40	0.01914	7.70	0.00435	7.20	0.00033
CUST_157_PI424980000	AAEL001320-RA	cytochrome p450 (CYP9M4)	4.10	0.01679	13.20	0.00317	7.20	0.00174
CUST_106_PI424980000	AAEL014616-RA	cytochrome p450 (CYP9J27)	3.70	0.01236	13.80	0.00178	7.80	0.00022
CUST_140_PI424980000	AAEL014614-RA	cytochrome p450 (as CYP9J4 in *An. gambiae*)	2.10	0.00352	13.80	0.00178	2.90	0.00104
CUST_228_PI424980000	AAEL004724-RA	carboxylesterase	2.00	0.00243	2.10	0.00808	2.80	0.00046
Common to KB and KL but not PG								
CUST_151_PI424980000	AAEL001288-RA	cytochrome p450 (as CYP9M1 in *An. gambiae*)	2.50	0.00908	2.40	0.00835		
Common to KB and PG but not KL								
CUST_10688_PI424980000	AAEL012836-RA	cytochrome b561	4.40	0.00986			9.50	0.00022
CUST_67_PI424980000	AAEL014893-RA	cytochrome p450 (CYP6BB2)	2.00	0.00104			5.20	0.00019
Common to KL and PG but not KB								
CUST_162_PI424980000	AAEL014607-RA	cytochrome p450 (CYP9J26)			356.70	0.00214	395.70	0.00022
CUST_145_PI424980000	AAEL014606-RA	cytochrome p450 (CYP9J27)			16.90	0.00349	12.20	0.00069
CUST_131_PI424980000	AAEL008023-RA	cytochrome p450 (CYP4C52)			12.70	0.00512	3.50	0.00376
CUST_148_PI424980000	AAEL014891-RA	cytochrome p450 (as CYP6P4 in *An. gambiae*)			8.50	0.00978	9.70	0.00117
CUST_64_PI424980000	AAEL007473-RA	cytochrome p450 (CYP6AH1)			5.90	0.00752	2.50	0.0024
CUST_176_PI424980000	AAEL007951-RA	glutathione-s-transferase gst			4.50	0.00397	2.40	0.00291

		(GSTE2)						
CUST_256_PI424980000	AAEL004118-RA	aldo-keto reductase			3.20	0.00252	5.00	0.0006
CUST_352_PI424980000	AAEL008672-RA	abc transporter			2.50	0.00711	3.40	0.00056
		KB only						
CUST_8318_PI424980000	AAEL014246-RA	glucosyl glucuronosyl transferases	3.60	0.04007				
CUST_348_PI424980000	AAEL004331-RA	abc transporter	3.20	0.00636				
CUST_112_PI424980000	AAEL001292-RA	cytochrome p450 (CYP9M7)	3.00	0.03684				
		KL only						
CUST_143_PI424980000	AAEL014617-RA	cytochrome p450 (CYP9J28)			10.80	0.00209		
CUST_1768_PI424980000	AAEL010590-RA	aldose 1-epimerase			5.70	0.00216		
CUST_6_PI424980000	AAEL004870-RA	cytochrome p450 (CYP18A1)			3.20	0.00725		
CUST_7415_PI424980000	AAEL010157-RA	microsomal glutathione s-transferase			2.80	0.00212		
CUST_824_PI424980000	AAEL005188-RA	alpha-n-acetyl galactosaminidase			2.70	0.00349		
CUST_111_PI424980000	AAEL001312-RA	cytochrome p450 (CYP9M6)			2.40	0.00402		
CUST_9474_PI424980000	AAEL007674-RA	alpha-amylase			2.30	0.00413		
		PG only						
CUST_88_PI424980000	AAEL012491-RA	cytochrome p450 (CYP6P12)					6.50	0.00091
CUST_67_PI424980000	AAEL014893-RA	cytochrome p450 (CYP6BB2)					5.20	0.00019
CUST_86_PI424980000	AAEL009126-RA	cytochrome p450 (CYP6N6)					4.90	0.00041
CUST_129_PI424980000	AAEL005695-RA	cytochrome p450 (CYP325X1)					4.60	0.00542
CUST_184_PI424980000	AAEL011741-RB	glutathione s-transferase (GSTS1)					4.30	0.00456
CUST_233_PI424980000	AAEL000905-RA	carboxylesterase					4.00	0.00082
CUST_3471_PI424980000	AAEL005937-RA	atp-binding cassette transporter					3.60	0.00039
CUST_233_PI424980000	AAEL000905-RA	carboxylesterase					4.00	0.00082

CUST_44_PI424980000	AAEL007795-RA	cytochrome p450 (CYP4D37)	3.40	0.00936
CUST_338_PI424980000	AAEL008624-RA	abc transporter	3.40	0.00029
CUST_87_PI424980000	AAEL009121-RA	cytochrome p450 (CYP6N9)	3.50	0.00045
CUST_44_PI424980000	AAEL007795-RA	cytochrome p450 (CYP4D37)	3.40	0.00936
CUST_31_PI424980000	AAEL017136-RA	cytochrome p450 (CYP325V1)	3.10	0.00046
CUST_95_PI424980000	AAEL006798-RA	cytochrome p450 (CYP9J10)	3.00	0.00064
CUST_2985_PI424980000	AAEL013458-RB	glutamine synthetase 2	3.00	0.00111
CUST_2984_PI424980000	AAEL013458-RA	glutamine synthetase 2	2.90	0.0009
CUST_11833_PI424980000	AAEL014612-RA	cytochrome p450 (as CYP9J5 in *An. gambiae*)	2.90	0.00109
CUST_93_PI424980000	AAEL009131-RA	cytochrome p450 (CYP6Z8)	2.50	0.00639
CUST_9043_PI424980000	AAEL011981-RA	glutamate decarboxylase	2.60	0.00026
CUST_6733_PI424980000	AAEL007523-RA	spermidine oxidase	2.40	0.00162
CUST_6046_PI424980000	AAEL009119-RA	cytochrome p450 (as CYP6M2 in *An. gambiae*)	2.40	0.00168
CUST_10102_PI424980000	AAEL012838-RA	cytochrome b561 as CYP325K1 in *An. gambiae*)	2.30	0.00055
CUST_130_PI424980000	AAEL008017-RA	cytochrome p450 (CYP4C50)	2.30	0.00943
CUST_102_PI424980000	AAEL017217-RA	cytochrome p450 (as CYP9J5 in *An. gambiae*)	2.30	0.00039
CUST_7589_PI424980000	AAEL015641-RA	cytochrome p450 (as CYP6AH1 in *An. gambiae*)	2.20	0.00047
CUST_108_PI424980000	AAEL002638-RA	cytochrome p450 (CYP9J6)	2.20	0.00046
CUST_4_PI424980000	AAEL002031-RA	cytochrome p450 (CYP12F7)	2.10	0.00123
CUST_346_PI424980000	AAEL006717-RA	abc transporter	2.10	0.00138
CUST_194_PI424980000	AAEL002367-RA	Carboxylesterase (CCEAE1B)	2.10	0.00228
CUST_357_PI424980000	AAEL005249-RA	abc transporter	2.00	0.00778

Table 4.15 Probes from detoxification genes & genes linked with resistance down-regulated in *Ae. aegypti* in all three locations in comparison with susceptible New Orleans strain. FC = fold change. (p = 0.01)

Probe Name	Systematic Name	Blast2GO Annotation	Kota Bharu vs NO		Kuala Lumpur vs NO		Penang vs NO	
			Absolute FC	Corrected p-value	Absolute FC	Corrected p-value	Absolute FC	Corrected p-value
Common to all three locations								
CUST_22_PI424980000	AAEL012769-RA	cytochrome p450 (CYP325M2)	2.95	0.006449	5.36	0.011548	6.39	9.43E-04
CUST_12428_PI424980000	AAEL015476-RA	cytochrome p450 (as CYP325F2 in *An. gambiae*)	2.35	7.53E-04			4.41	0.005393
CUST_18_PI424980000	AAEL012766-RA	cytochrome p450 (CYP325G2)	2.17	0.017221			13.90	3.65E-04
Common to KB and PG but not KL								
CUST_4119_PI424980000	AAEL015475-RA	cytochrome p450 (as CYP325C2 in *An. gambiae*)			4.39	0.003937	15.83	0.001003
CUST_55_PI424980000	AAEL014019-RB	cytochrome p450 (CYP4J16)			4.09	0.007248	6.33	0.004038
CUST_128_PI424980000	AAEL012761-RA	cytochrome p450 (CYP325T2)			3.37	0.00709	9.06	7.90E-04
KL only								
CUST_127_PI424980000	AAEL000325-RA	cytochrome p450 (as CYP325C3 in *An. gambiae*)			3.48	0.010751		
CUST_11713_PI424980000	AAEL005375-RA	glucosyl glucuronosyl transferases			2.07	0.024392		
CUST_2203_PI424980000	AAEL009298-RA	n-acetylneuraminate lyase			2.61	0.009488		
CUST_2240_PI424980000	AAEL012764-RA	glycine n-methyltransferase			2.60	0.008627		
CUST_54_PI424980000	AAEL013554-RA	cytochrome p450 (CYP4J14)			2.58	0.006615		
CUST_133_PI424980000	AAEL013556-RA	cytochrome p450 (CYP4J15)			2.26	0.008072		
PG only								
CUST_32_PI424980000	AAEL005696-RA	cytochrome p450 (CYP325X2)					26.97	1.72E-04
CUST_104_PI424980000	AAEL014615-RA	cytochrome p450 (CYP9J23)					12.18	0.003133
CUST_175_PI424980000	AAEL007954-RA	glutathione-s-transferase (GSTE1)					8.43	0.002579
CUST_25_PI424980000	AAEL012770-RA	cytochrome p450 (CYP32N1)					6.42	4.28E-04
CUST_136_PI424980000	AAEL014680-RA	cytochrome p450 (CYP6CA1)					6.32	2.93E-04

CUST_68_PI424980000	AAEL017539-RA	cytochrome p450 (CYP6BY1)	5.85	5.01E-04
CUST_83_PI424980000	AAEL009133-RA	cytochrome p450 (CYP6N14)	4.77	0.004511
CUST_138_PI424980000	AAEL009122-RA	cytochrome p450 (as CYP6N2 in *An. gambiae*)	4.53	0.005026
CUST_53_PI424980000	AAEL013555-RA	cytochrome p450 (CYP4J13)	4.31	2.81E-04
CUST_56_PI424980000	AAEL014019-RA	cytochrome p450 (CYP4J16)	4.27	5.49E-04
CUST_117_PI424980000	AAEL002067-RA	cytochrome p450 (as CYP15B1 in *An. gambiae*)	3.59	8.86E-04
CUST_342_PI424980000	AAEL014019-RA	cytochrome p450 (CYP4J16)	3.58	0.00418
CUST_139_PI424980000	AAEL003748-RA	cytochrome p450 (CYP9AE1)	3.47	2.81E-04
CUST_49_PI424980000	AAEL003380-RA	cytochrome p450 (CYP4H28)	3.37	7.37E-04
CUST_3780_PI424980000	AAEL014830-RA	cytochrome p450 (as CYP4J10 in *An. gambiae*)	3.25	9.39E-04
CUST_2203_PI424980000	AAEL009298-RA	n-acetylneuraminate lyase	2.87	0.001276
CUST_89_PI424980000	AAEL009120-RA	cytochrome p450 (CYP6S3)	2.82	8.19E-04
CUST_118_PI424980000	AAEL011463-RA	cytochrome p450 (as CYP302A1 in *An. gambiae*)	2.69	9.83E-04
CUST_355_PI424980000	AAEL008635-RA	abc transporter	2.67	4.52E-04
CUST_39_PI424980000	AAEL010154-RA	cytochrome p450 (CYP4AR2)	2.57	0.004548
CUST_63_PI424980000	AAEL015654-RA	cytochrome p450 (CYP6AG8)	2.57	0.001943
CUST_54_PI424980000	AAEL013554-RA	cytochrome p450 (CYP4J14)	2.56	3.26E-04
CUST_125_PI424980000	AAEL006058-RA	cytochrome p450 (CYP325Q2)	2.53	0.003763
CUST_20_PI424980000	AAEL005788-RA	cytochrome p450 (CYP325K3)	2.51	0.00114
CUST_8_PI424980000	AAEL015655-RA	cytochrome p450 (CYP302A1)	2.24	0.001686
CUST_9_PI424980000	AAEL014412-RA	cytochrome p450 (CYP304B2)	2.23	0.003468
CUST_196_PI424980000	AAEL002385-RA	Carboxylesterase (CCEAE3B)	2.23	5.35E-04
CUST_2721_PI424980000	AAEL008663-RA	aldo-keto reductase	2.20	0.009233
CUST_82_PI424980000	AAEL009137-RA	cytochrome p450 (CYP6N13)	2.18	0.001756
CUST_85_PI424980000	AAEL010158-RA	cytochrome p450 (CYP6N17)	2.16	0.008654

4.3.8 Validation of candidate genes through qRT-PCR

After conducting the analysis of the microarray data using the Genespring 12.0 software, 7 candidate genes were chosen out of the 245 commonly transcribed genes. The genes were Trypsin, Multisynthetase complex, CYP6CB1, CYP9J26-609 (AAEL014609-RA), CYP9J26-607 (AAEL014607-RA), CYP9M4 and CYP9J27. These genes chosen were significantly up-regulated in all three (or two) field populations. Out of the 7 genes, 5 were cyctochrome P450s (Table 4.14).

The most over-expressed P450 for microarray in all 3 locations, CYP6CB1 was not significantly over-expressed from the qRT-PCR results in all four locations including JB (Figure 4.18). However, the over-expression of other genes was confirmed apart from the multisynthetase gene. The JB sample consistently exhibited a lower expression level for all these genes in comparison to the other 3 locations. This difference could be associated with the lower resistance level to pyrethroids and DDT in JB.

Overall, for most of the genes, the highest over-expression was observed for the KL population which correlates with the high resistance level observed in KL from bioassays. The expression profile of the five significantly over-expressed genes from qRT-PCR was further assessed for various samples from mosquitoes alive after exposure to different insecticides. From this analysis, the CYP9J27 gene was more over-expressed, although not significantly, in the DDT resistant mosquitoes from PG than the other insecticides (Figure 4.19). CYP9M4 was significantly more over-expressed in the PG bendiocarb resistant sample than other mosquito samples. No significant difference was observed between samples for the two CYP9J26 P450 genes and for the trypsin gene.

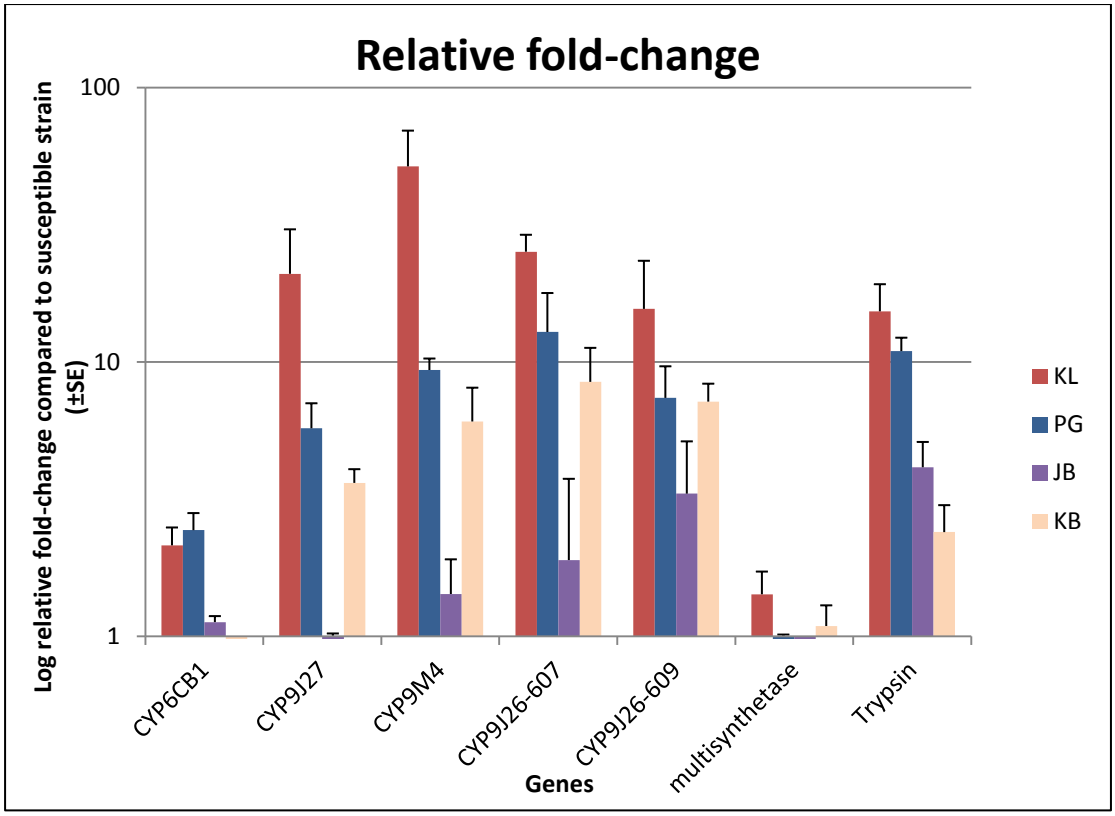

Figure 4.18 Relative fold-change of candidate genes in *Ae. aegypti* from qRT-PCR analysis.

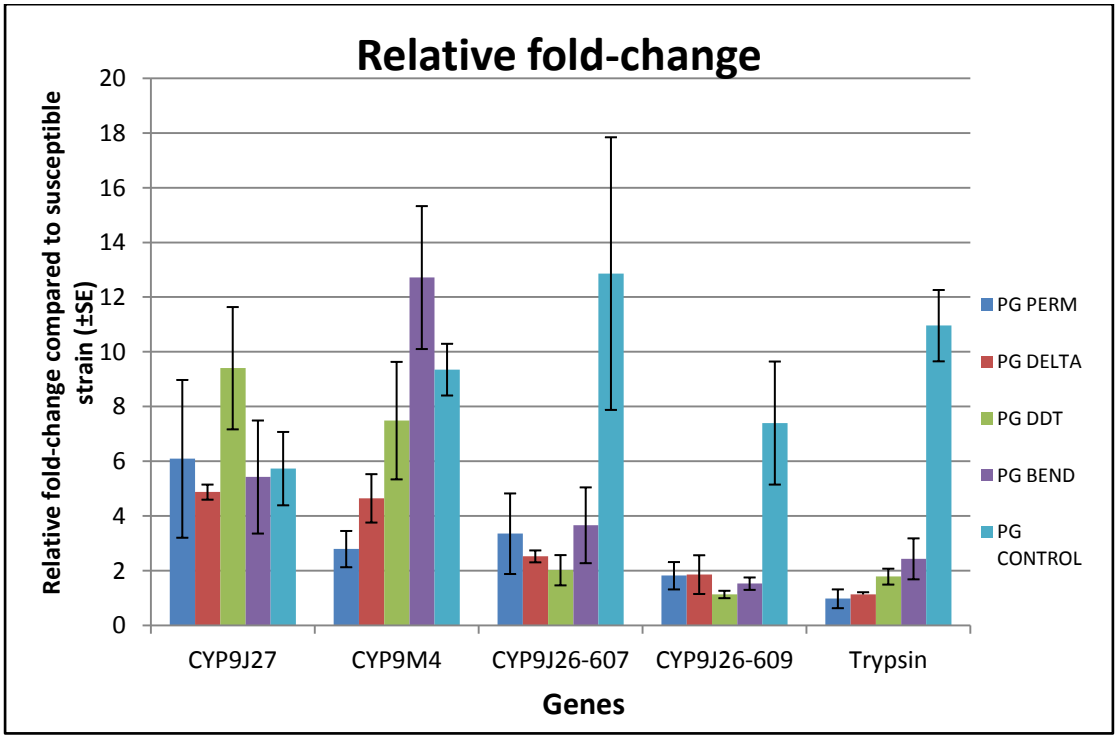

Figure 4.19 Relative fold-change of candidate genes in *Ae. aegypti* from qRT-PCR analysis for resistant samples from Penang.

4.3.9 GO Enrichment analysis

GO Enrichment analysis was used to identify particular Gene Ontology (GO) terms that were over represented in the data set of transcripts up regulated in all three resistant populations and in single populations.

When comparing the commonly up-regulated genes in all three populations at p=0.01, a few GO terms that relates to detoxification was observed. Among which was NADPH-hemoprotein reductase activity, ATP binding and others (Figure 4.18).

When observing the GO terms of single locations either in PG (Figure 4.19), KB (Figure 4.20) or KL (Figure 4.21), interesting terms such as ATP binding, heme binding and monooxigenase activity were up-regulated possibly associated with the multiple resistance to insecticides detected in these locations.

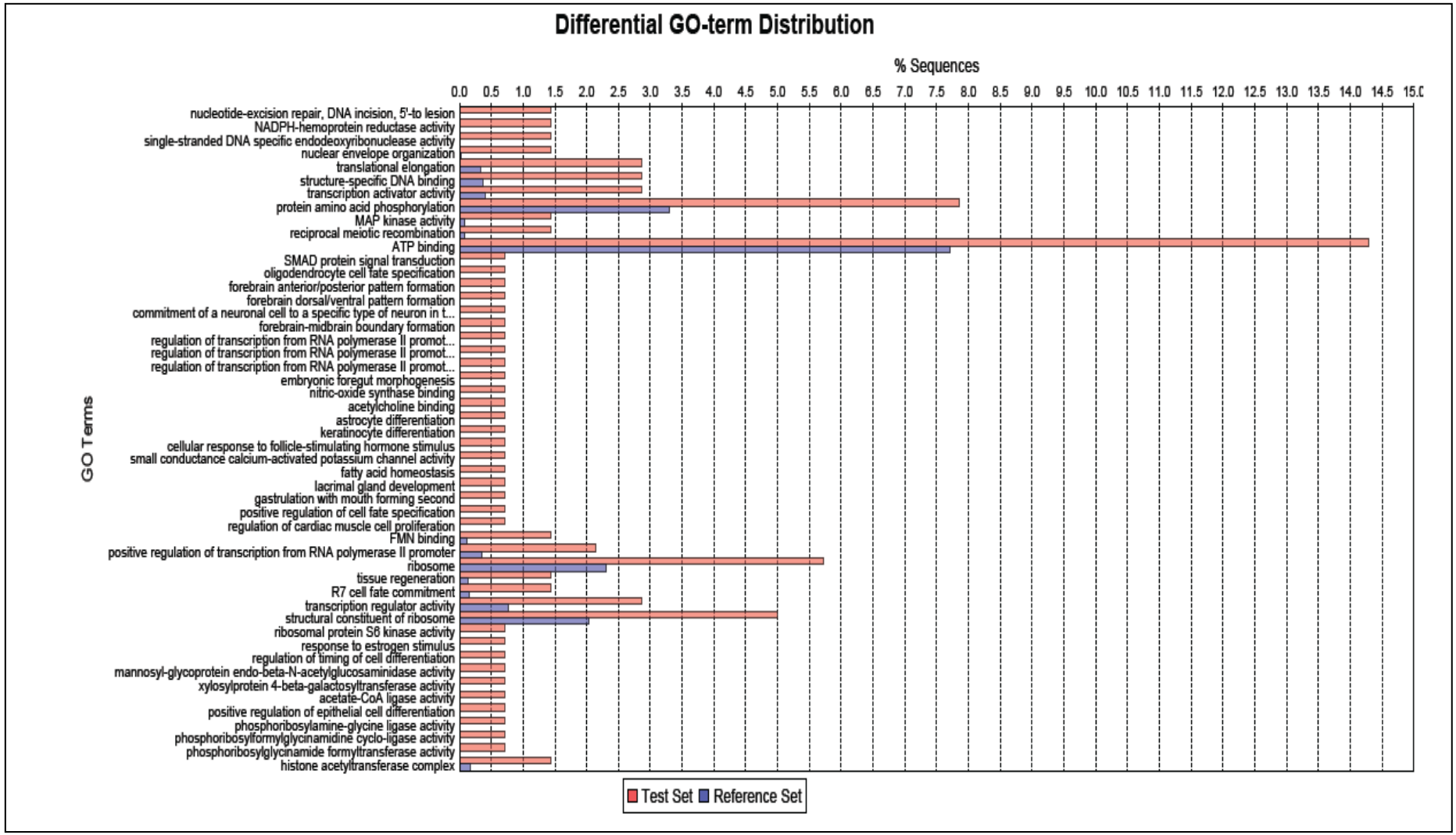

Figure 4.20 GO analysis of common genes in *Ae. aegypti* in Penang, Kuala Lumpur and Kota Bharu.

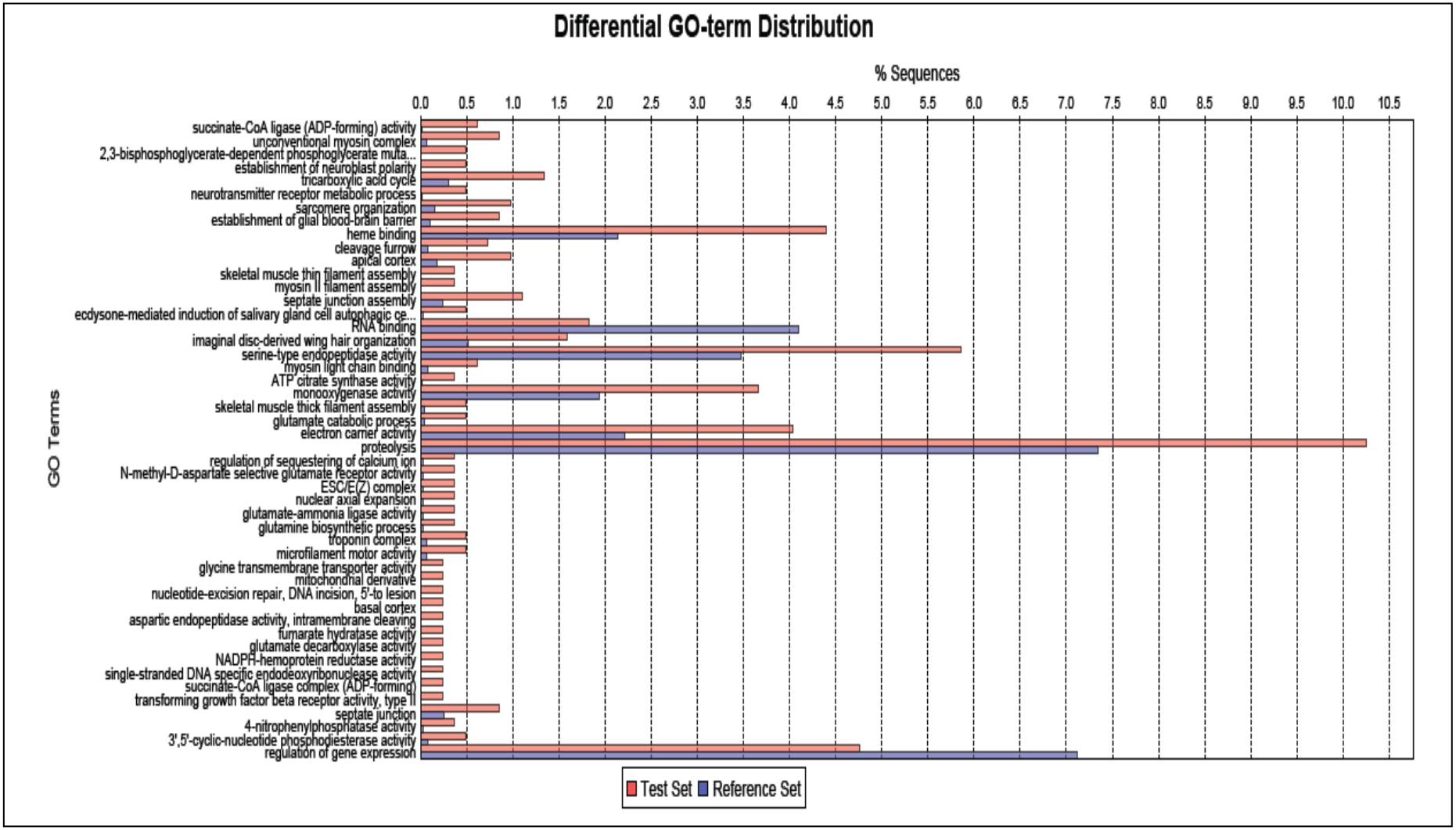

Figure 4.21 GO analysis of genes in *Ae. aegypti* in Penang.

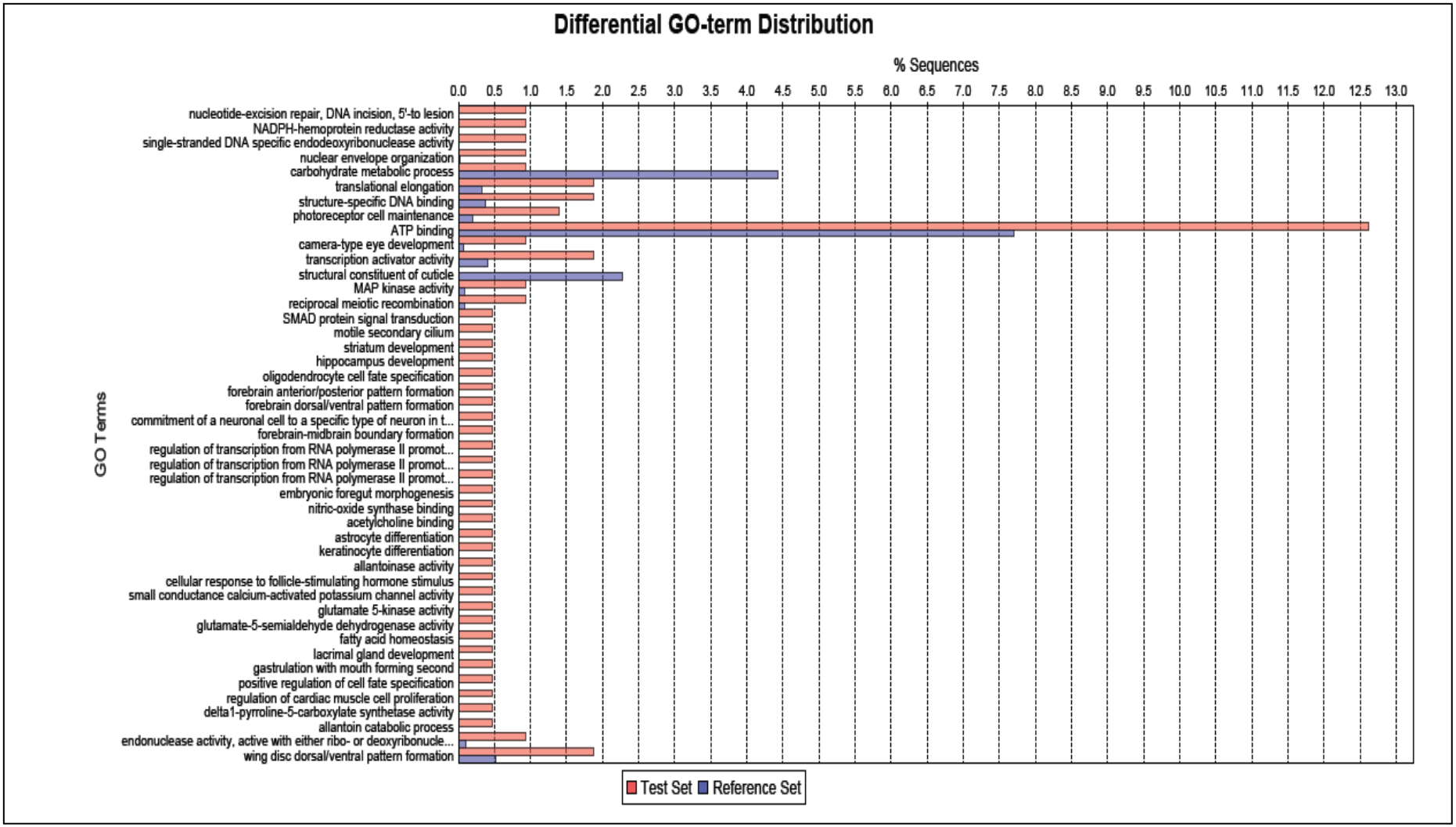

Figure 4.22 GO analysis of genes in *Ae. aegypti* in Kota Bharu.

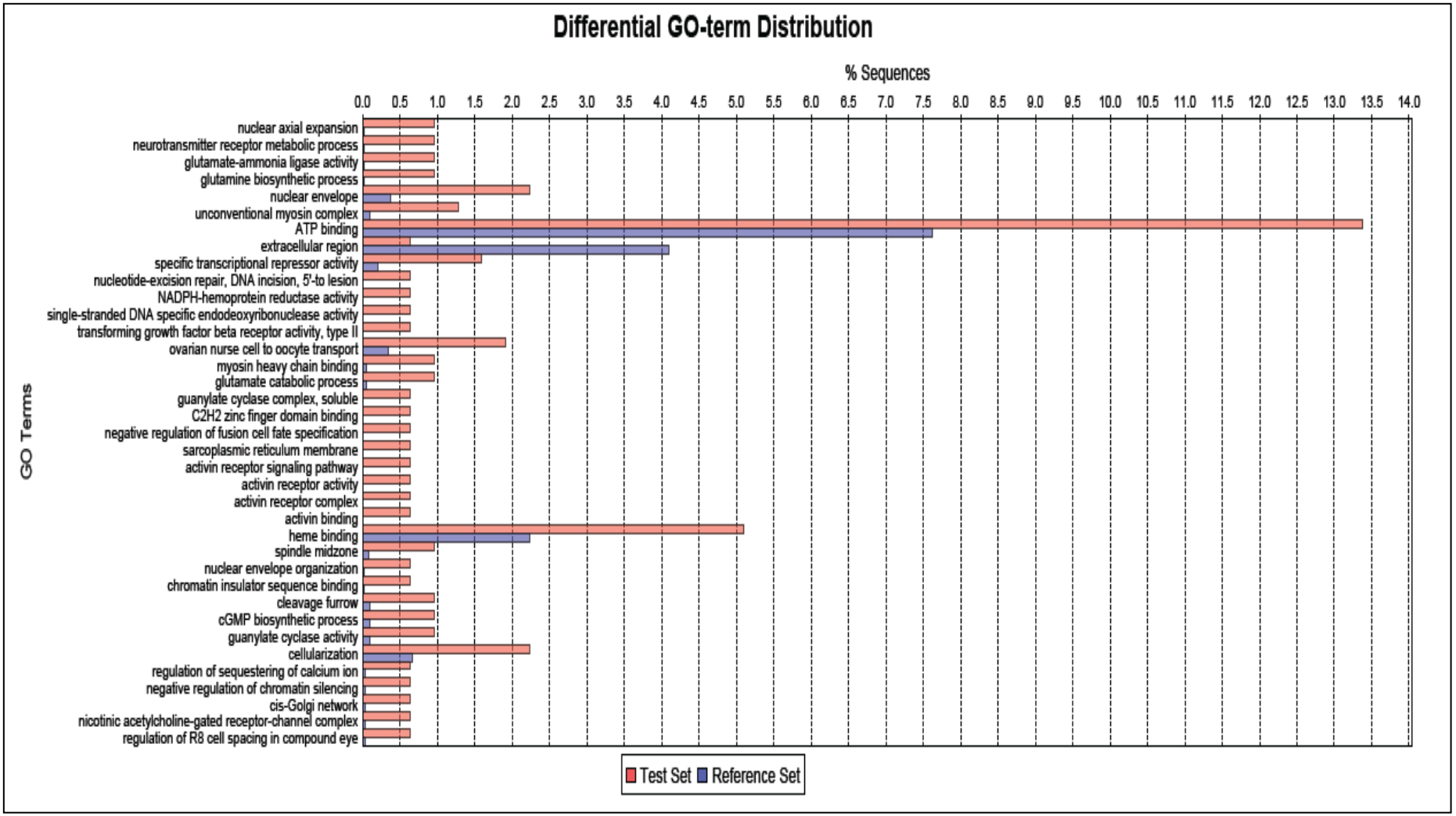

Figure 4.23 GO analysis of genes in *Ae. aegypti* in Kuala Lumpur.

4.4 Discussion

This study has focused on the characterisation of mechanism conferring resistance to insecticides in Malaysian population of *Ae. aegypti*. This work was among the first to compare the resistance profile as well as establishing the contribution of target-site resistance and metabolic resistance in the resistance to insecticides observed in *Ae. aegypti* populations across Malaysia.

As there are two dengue vectors in Malaysia namely *Ae. aegypti* and *Ae. albopictus*, the PCR identification of the field samples using the PCR based method provided a great confidence in the species composition of the of the samples collected (Beebe *et al.*, 2007). This species identification confirmed that both dengue vectors were sympatric in the four locations and that this co-occurrence should be taken into account in the implementation of dengue control interventions in Malaysia.

Presence of knockdown resistance mechanisms in *Ae. aegypti* populations across Malaysia

Since there was a high level of pyrethroid and DDT resistance in all of the four strains, the presence of knockdown resistance (*kdr*) mutations were investigated across *Ae. aegypti* populations from Malaysia and their potential association with resistance assessed. Pyrosequencing genotyping of the three commonly detected mutations *kdr*1011, *kdr*1016 and *kdr*1534, which have been reported to confer resistance in *Ae. aegypti* (Saavedra-Rodriguez *et al*., 2007, Harris *et al*., 2010) only successfully detected the F1534C mutation. Despite repeated attempts, the pyrosequencing could not detect the V1016G mutation although it was later detected by cDNA sequencing. The inability of pyrosequencing to genotype this mutation highlights the needs for confirmation of such detection with other genotyping methods such as the melt-curve method used in this study. The presence of F1534C mutation was the first report of *kdr* resistance in the Malaysian population. However, this mutation has already been seen in *Ae.*

aegypti populations around the world; in Latin America (Brengues *et al.*, 2003),Thailand (Saavedra-Rodriguez *et al.*, 2007) and in Cayman Island (Harris *et al.*, 2010). The F1534C mutation has also been reported in *Ae. albopictus* from Singapore (Kasai *et al.*, 2011).

Analysis of the correlation between the genotypes of the F1534C mutation and resistance to pyrethroids and DDT showed that it did not always establish the presence of such correlation. In some location such as in PG, a significant correlation was established for both type I (permethrin) and type II (deltamethrin) pyrethroids. Such correlation supports that the F1534C was playing a role in the pyrethroid resistance in PG similar to previous findings in other strains of this species (Harris *et al*., 2010, Vontas *et al*., 2012). However, such correlation was not observed in other location such as in KB suggesting that presence of the F1534C mutation does not automatically result to resistance to pyrethroids or that other mechanisms such as metabolic resistance are playing a more predominant role in the resistance observed in such locations. Correlation of the F1534C with DDT resistance could not be properly assessed in most of the locations because of the low number of susceptible mosquitoes. However in KB where such assessment was possible, the correlation was not significant although a trend was present (OR = 2.65, P = 0.078). The presence of both susceptible and resistance alleles in DDT resistance mosquitoes suggests that if this mutation is playing a role in the DDT resistance, it will be along other mechanisms such as metabolic resistance. Overall, the analysis of the polymorphism pattern of intron 27 to exon 31 spanning the F1534C mutation supported that this mutation was playing a role in permethrin resistance across Malaysia with a predominance of a resistant haplotype. This reduced diversity around this VGSC fragment was similar to cases observed around the L1014F mutation in *An. gambiae* (Pinto *et al.*, 2007) where a major resistant haplotype was detected. However, it cannot be ruled out that this reduced genetic diversity of the VGSC gene is not also associated with other *kdr* mutation than F1534C. Further analysis of polymorphism of this gene across Malaysia will help to

confirm the exact contribution of knockdown resistance in the pyrethroid and DDT resistance observed.

The sequencing of cDNA of the VGSC gene further detected the V1016G mutation. However, this mutation was present at a lower frequency than the F1534C suggesting that it was more recent in Malaysia either through migration from neighbouring countries or independent occurrence. The V1016G has previously been detected in other countries in the region such as in an *Ae. aegypti* strain from Indonesia (Brengues *et al.*, 2003) and in Taiwan (Chang *et al.*, 2009). However, no significant correlation could be observed between the V1016G and the three insecticides tested (permethrin, deltamethrin and DDT) suggesting that this mutation may only be playing a minor role in the observed resistance.

The presence of other target site resistance was also investigated with the sequencing of the Acetylcholinesterase (*Ace-1*) gene which could harbour mutations conferring resistance against carbamates and organophosphates. However, the experiment failed due to the presence of indels in the sequences that were obtained. To overcome this problem, the next step should be cloning of the sequences to obtain good sequences. Mutation in the *Ace-1* gene has been observed in *An. gambiae* (Weill *et al.*, 2004) but none has been reported in *Ae. aegypti* worldwide (Vontas *et al.*, 2012).

Another target site resistance mechanism that could be explored was the possible presence of RDL mutation as dieldrin resistance was present in the Johor Bharu *Ae. aegypti* populations and as observed in other populations of this species such as in Puerto Rico (Khan and Brown, 1961). This mutation had also been reported to be found in *Cx. quinquefasciatus* and *Ae. albopictus* species in La Reunion Island (Tantely *et al.*, 2010).

Metabolic resistance mechanism in *Ae. aegypti* in Malaysia

The genome-wide transcriptional analyses carried out using microarray showed that the metabolic resistance plays an important role in conferring resistance to insecticides in *Ae. aegypti* across Malaysia. This further supports the synergist assay result with PBO showing a recovery of susceptibility notably for pyrethroids. The role of metabolic resistance was supported by the over-expression of many genes belonging to detoxification gene families in PG, KL and KB when comparing them to the laboratory susceptible NO strain. The most preeminent detoxification gene family was the cytochrome P450 genes which were the only detoxification family except for one unique carboxylesterase commonly over-expressed in the three locations.

The most over-expressed cytochrome P450 is the CYP6CB1 which has also been reported in a strain from Isla Mujeres in Mexico (Stevenson *et al.*, 2012). Unfortunately, this microarray over-expression was not supported by the qRT-PCR validation for all four populations tested. This discrepancy between the two methods could be caused by differences in the sequences of the microarray probes and the qRT-PCR primers. However, recent functional analysis had shown that this CYP6CB1 gene could not metabolise pyrethroids (Stevenson *et al.*, 2012) although it could still do so for other insecticides. CYP9J26 (AAEL014609) gene was among the top up-regulated detoxification gene which has also been reported in Cuba, Thailand and Grand Cayman and has also been functionally validated to confer pyrethroid resistance (Stevenson *et al.*, 2012).

Overall, several P450 genes belonging to the CYP9 family were over-expressed in the C-S across Malaysia including two transcripts of CYP9J26, CYP9J27, CYP9J28, CYP9M6 while only few cytochrome P450s from the CYP6 family (CYP6P12, CYP6BB2) were over-expressed and usually at lower fold change. This was further supported by previous studies worldwide showing that contrary to *Anopheles* mosquitoes, genes from the CYP9 family play a more important role than those from the CYP6 family in insecticide resistance in *Ae. aegypti*

(Strode *et al*., 2008, Marcombe *et al*., 2009, Bariami *et al*., 2012, Vontas *et al*., 2012).

Interestingly, the top most commonly over-expressed gene was the anionic-trypsin which is found in the midgut of mosquitoes to hydrolyse proteins after blood meals. This serine proteinase was found to be over-expressed in deltamethrin resistant strain of *Culex pipiens pallens* from China (Gong *et al.*, 2005). Alarmingly, the amount of trypsin in the dengue vector midgut could also determine the infection and replication rate of DEN-2 virus (Molina-Cruz *et al.*, 2005).

Not many glutathione-S transferases were detected compared to cytochrome P450s despite the very high DDT resistance notably in KL. The PBO synergist assay indicated a recovery of susceptibility from 0 to 35% in KL for DDT. The low expression of GST notably that of the known DDT metaboliser GSTe2 (FC = 4.5) (Lumjuan *et al.*, 2011) shows that knockdown resistance could be responsible for most of the remaining 65% lost of DDT susceptibility. Similar assessment of pyrethroids shows a recovery of susceptibility after PBO assay from 1% to 26% for permethrin and from 0% to 71% for deltamethrin. This suggests that metabolic resistance through P450 up-regulation was more important for deltamethrin than permethrin resistance and *kdr* playing a more important role for permethrin than deltamethrin. This will be in line with the higher correlation observed between permethrin and F1534C genotypes than with deltamethrin.

PBO assays with bendiocarb led to a nearly full recovery of the susceptibility to this insecticide suggesting that some of the cytochrome P450 genes detected in this study are mainly responsible for this resistance. This will explain the low expression of carboxylesterase genes and suggest an absence of any *Ace-1* mutations as previously reported (Vontas *et al.*, 2012).

Conclusion

From this study, we can observe that both target site resistance and metabolic resistance mechanism play a role in the resistance of the *Ae. aegypti* population in Malaysia. The F1534C *kdr* mutation contributes to the high pyrethroid and DDT resistance while this was not established for the V1016G mutation. The involvement of cytochrome P450 genes in the resistance of other insecticide is also important. However, further functional characterisation work using either transgenic expression in *Drosophila* flies or recombinant enzymes in *E. coli* has to be done to confirm the exact contribution of these candidate genes in the resistance profile observed in *Ae. aegypti* populations across Malaysia.

It is a worrying sign that the control of *Ae. aegypti* is no longer fully effective by using pyrethroids (which is the main insecticide in dengue intervention control program) due to the high level of resistance. An alternative to pyrethroid is the organophosphate, malathion since all of the populations are susceptible to this insecticide. However, proper management has to be conducted to prevent a more serious resistance problem from occurring in Malaysia.

5.0 INSECTICIDE RESISTANCE MECHANISM IN *AEDES ALBOPICTUS IN MALAYSIA*

5.1 Introduction

Dengue fever is transmitted by the *Aedes* mosquito and among them is the *Ae. albopictus* Skuse. Although it has lower oral receptivity for dengue virus compared with *Ae. aegypti (Vazeille et al., 2003)* it plays an important role in maintaining the dengue virus by transmitting the virus through mating and transovarial transmission (Rosen et al., 1983, Rosen, 1987). In the absence of *Ae. aegypti*, *Ae. albopictus* could also cause dengue outbreaks (Gratz, 2004). Also, as reported by Leroy *et al.* (2009) *Ae. albopictus* was also involved in chikungunya fever in Madagascar. In China, this species was also the cause of dengue outbreaks in 2004 (Xu *et al.*, 2007).

Since both *Aedes* species are vectors of dengue fever, efforts are always implemented to control the number of dengue cases and the most effective way is by using chemical control. Chemical control using insecticides in ULV space sprays, fogging and thermal spraying has caused the emergence of resistance (Hidayati *et al.*, 2005). The reliance on only a few insecticide classes which is suitable for public health use has added to the cause of resistance. Similarly to the case of *Ae. aegypti*, *Ae. albopictus* has also developed resistance towards insecticides (Hidayati *et al.*, 2005). The underlying resistance mechanism in *Ae. albopictus* is less explored compared to *Ae. aegypti*.

Aedes albopictus was originally considered to be a species from South East Asia. Unfortunately, due to the expansion of technology trough transportation and trades between the east and the west, this species was able to be spread to other continents such as Europe, Africa and America (Gratz, 2004, Vontas et al., 2012). In some countries, Ae. albopictus has developed resistance towards some insecticides and has caused problems in vector control programs (Lambrechts *et al.*, 2010). Resistance towards DDT has been recorded in adult *Ae. albopictus* populations from Cameroon (Kamgang *et al.*, 2011) and Sri Lanka (Dharshini *et*

al., 2011) with mortality of less than 50% after 1 hour exposure to 4% DDT (Ranson et al., 2010, Vontas et al., 2012). In Sri Lanka, adult *Ae. albopictus* population were moderately resistant towards 0.8% malathion with 55% mortality. Resistance toward carbamate (propoxur) and pyrethroids either permethrin or deltamethrin have not been recorded in adult populations from India, Thailand Greece and Italy (Vontas *et al.*, 2012).

In Malaysia permethrin is the main or common insecticide used for mosquito control. Although it has been used for a while now, the cases of permethrin resistance have not been reported for *Aedes* mosquitoes in Malaysia. Rohani *et al.* (2001) showed that the resistance of *Ae. albopictus* to permethrin was low. Another study conducted in Kuala Lumpur showed that this species was susceptible to permethrin (Wan-Norafikah *et al.*, 2013). Only very recently a study conducted by Chan & Zairi (2013) showed the presence of resistance in *Ae. albopictus* larvae towards permethrin and deltamethrin in Malaysia. Their study ruled out mutation in the voltage gated sodium channel gene and concluded that the resistance was caused by cytochrome P450 monooxygenase after conducting synergism assays (Chan and Zairi, 2013).

Elucidation of underlying resistance mechanisms are less advanced for *Ae. albopictus* than *Ae. aegypti* worldwide. It could probably be due to *Ae. albopictus* being less involved in cases of dengue fever or chikungunya fever. Another reason could be because less progress has been made in the genetics and genomics of this vector than for *Ae. aegypti*. For example, the genome of *Ae. albopictus* is still not published in contrast to that of *Ae. aegypti* which was published in 2007 (Nene *et al.*, 2007). However, this vector is becoming more noticed as a primary vector of these diseases in some countries instead of *Ae. aegypti* such as in La Réunion island (Delatte *et al.*, 2010). This is particularly true in Malaysia where no such study has been carried out. Studies of resistance through molecular techniques are less conducted in Malaysia.

This chapter investigates the molecular basis of insecticide resistance, with emphasis on permethrin resistance, in field populations of *Ae. albopictus* across

Malaysia. Potential role of target-site resistance and metabolic resistance mechanisms are investigated.

5.2 Materials and Methods

5.2.1 Mosquito samples

Details of field collections, rearing conditions and susceptibility bioassays are described in Chapter 2 and 3. Mosquitoes obtained from the bioassays were used for the molecular experiments described in this chapter.

5.2.2 Genomic DNA extraction and species identification

As mentioned in section 2.2, genomic DNA was extracted from 50 individual F_0 field mosquitoes for *Ae. albopictus* from Penang (PG), Kuala Lumpur (KL), Johor Bharu (JB) and Kota Bharu (KB) to confirm the species of the samples used. 30 out of 50 F_0 field mosquitoes were used for detection of k*dr* mutation.

5.2.3 Genotyping for target site resistance

Pyrosequencing genotyping and direct sequencing of the voltage gated sodium channel (VGSC) gene and the *Ace-1* gene were performed to detect the presence of target site resistance (*kdr* and *Ace-1* mutations) in *Ae. albopictus* in Malaysia.

5.2.3.1 Genotyping of known *kdr* mutations using pyrosequencing

Attempt was made to check whether the *kdr* mutations detected in *Ae. aegypti* were also present in *Ae. albopictus*. All four field populations of *Ae. albopictus* were genotyped using the pyrosequencing method to assess the presence of *kdr*

mutations. The preliminary detection of the *kdr* mutations was conducted on 30 F_0 samples (gDNA) to establish the frequency of the *kdr* mutations in all four populations.

The three primer sets used in Chapter 4 to detect three different *kdr* mutations *kdr*1011, *kdr*1016 and *kdr*1534 in *Ae. aegypti* were used (Table 2.1). The sequences of *Ae. aegypti* and *Ae. albopictus* VGSC gene exons were aligned using ClustalW on the EBI website to ensure that the primers used for *Ae. aegypti* could also be used for *Ae. albopictus*.

The pyrosequencing experiment was done according to the protocol described in Chapter 2. The results were then analysed by manually looking at the peaks that represents nucleotides conferring *kdr* genotype in the pyrograms (Figure 4.6).

5.2.3.2 Search for new *kdr* mutations through sequencing of the voltage gated sodium channel (VGSC)

Sequencing of the VGSC was carried out to detect the presence of other potential mutations. A fragment of the VGSC from exon 19 to exon 31 was amplified as these exons span the regions of the gene commonly associated with pyrethroid/DDT resistance. The primers used are listed in Table 2.2 and the amplification was done according to the conditions described in section 2.2.8.

The samples used are 3 DDT resistant mosquitoes from all four populations. Only DDT resistant samples were chosen since there was a higher resistance level in the *Ae. albopictus* samples tested compared to the pyrethroid resistance samples (only Penang and Kuala Lumpur showed moderate level of resistance). The samples used were in the form of cDNA obtained was from RNA extractions of pools of 10 adult female mosquitoes as described in Chapter 2.

All the amplified samples were purified and sent for direct-sequencing to Microgen, South Korea, and the sequences obtained were aligned using ClustalW (Thompson *et al.*, 1994) procedure implemented in Bioedit software.

5.2.3.3 Sequencing of *Ace-1* gene

Ace-1 gene was amplified because of the presence of both organophosphate (malathion) and carbamate (bendiocarb) resistance in the mosquitoes tested using adult bioassays. Three cDNA sets of Bendiocarb resistant samples from PG, KL and JB were amplified for the full-length of the *Ace-1* gene using the primers listed in Table 2.2 and methods described in section 2.2.10. The purified PCR products were sent for direct sequencing to Source BioScience LifeSciences, UK and the data obtained was aligned using ClustalW (Thompson *et al.*, 1994).

5.2.4 Investigating metabolic resistance using microarray

5.2.4.1 Microarray design

Microarray experiments were conducted to elucidate the molecular basis of metabolic resistance in *Ae. albopictus* populations across Malaysia. A genome-wide transcription profiling was carried out to detect the sets of genes differentially expressed in relation to resistance phenotypes possibly responsible for the metabolic resistance suggested from PBO synergist assays in *Ae. albopictus* populations throughout Malaysia. Total RNA were extracted from 3 replicates of pools of 10 adult female mosquitoes not exposed to insecticide [unexposed or Control (C)] from all four locations. A Malaysian laboratory susceptible strain (VCRU) was also used in the comparisons. Only samples from PG, KL and JB were used in the microarray. KB samples were omitted from the microarray comparisons due to the low resistance level in the population compared to the other strains. However, samples from KB were used in the validation of the candidate genes through qRT-PCR. The protocol for RNA extraction, cRNA labelling (Figure 4.1), microarray hybridization, scanning and statistical analysis is discussed in Chapter 2.

The microarray hybridization for *Ae. albopictus* was done using a new 8 x 60k Agilent *Ae. albopictus* chip designed by Charles Wondji. The chip contains 18600 *Ae. aegypti* transcripts (times 2 probes), 11500 *Ae. albopictus* RNA seq Expressed Sequence Tags (ESTs) (times 2 probes) and cytochrome P450 transcripts from Genbank (times 3 probes).

Two different experiments were designed to characterise resistance mechanisms in Malaysian *Ae. albopictus*. The first design (Figure 5.1) consisted in comparing control or non-exposed samples (C) from Penang, Kuala Lumpur and Johor Bharu against the susceptible VCRU lab strain. The aim through this design was to detect most of the genes associated with metabolic resistance to all insecticides in three locations. The second experiment was designed to further investigate mechanisms of permethrin resistance observed in Kuala Lumpur through three different comparisons; KL permethrin-resistant (alive after 1h exposure) against VCRU susceptible lab strain (R-S), KL non-exposed against VCRU (C-S) and KL permethrin-resistant against KL non-exposed (R-C) (Figure 5.2). This triangular design has been successfully used to detect pyrethroid resistance genes in the malaria vector *An. funestus* (Riveron *et al.*, 2013). The hybridisation parameters are the same as for *Ae. aegypti* (Section 2.2.4.3).

The arrays were scanned using an Agilent G2205B microarray scanner (Agilent Technologies, UK) using the default settings following the Agilent Microarray scanner System User Manual (v 7.0).

High (100% photo-multiplier tube (PMT)) and low (10% PMT) extended dynamic range (XDR) scan images were combined and extracted using the Agilent Feature Extraction (FE) software GE2_105_Jan09 (Agilent Technologies, UK) and the custom array grid template (028498_D_F_20100519.XML). Quality control (QC) reports were consulted to give an indication of array quality (Appendix 8.4). A QC score of 11/11 indicates that all 11 main array parameters were passed. QC parameters include signals from spike-in controls, spatial distribution of outliers and signals from non-control spots. A score between 8 and 11 is good and usable and anything below 8 leads indicates poor quality.

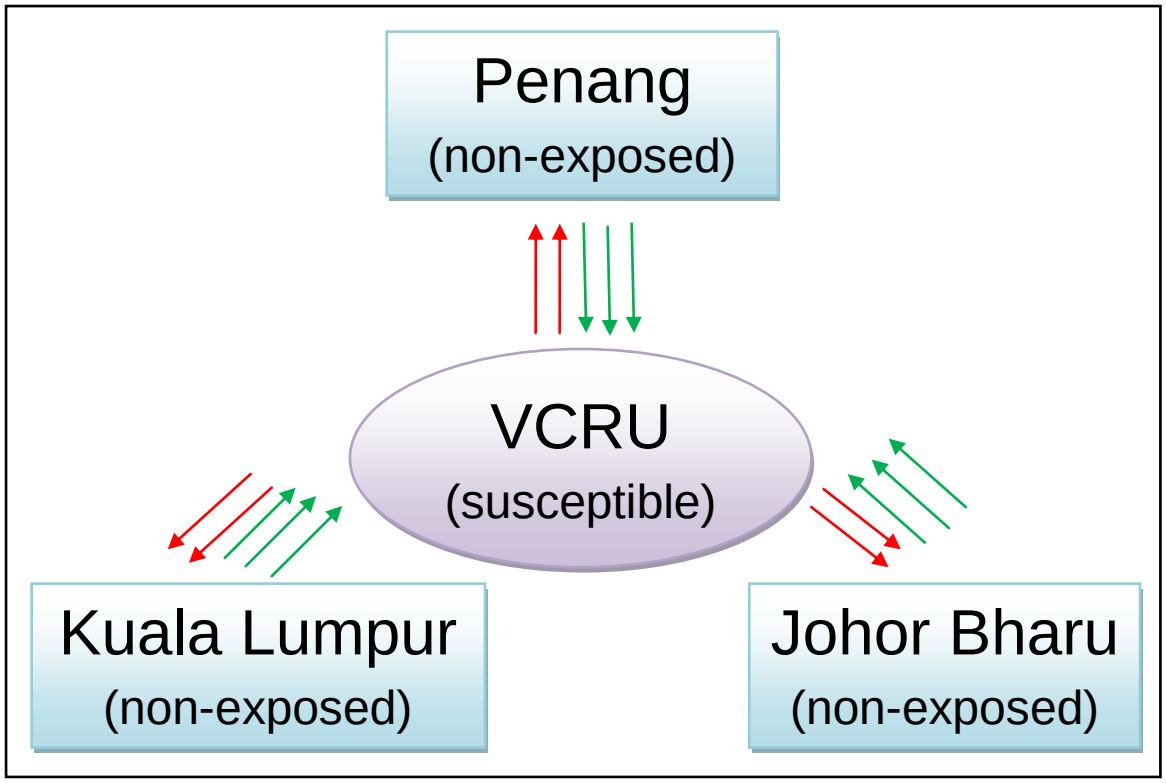

Figure 5.1 schematic representation of microarray design for *Aedes albopictus non-exposed vs susceptible samples.* Green arrows refer to Cy-3 dye, and red arrows refer to Cy-5 dye.

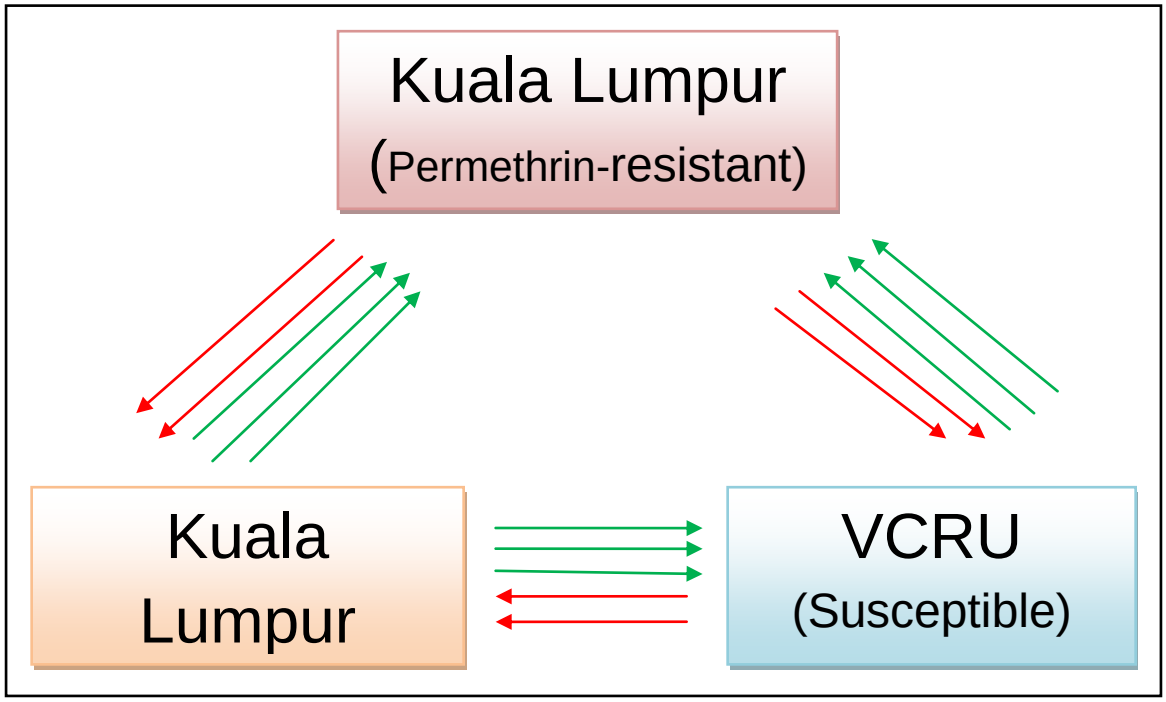

Figure 5.2 schematic representation of microarray design for *Aedes albopictus KL strain vs VCRU.* Green arrows refer to Cy-3 dye, and red arrows refer to Cy-5 dye.

5.2.4.2 Microarray data analysis

Genespring GX version 12 software (Agilent Technologies, US) was used to analyse the microarray data obtained. Mean expression ratios were assessed using a t-test against zero with a multiple testing correction (Benjamini-Hochberg false discovery rate). Genes showing both t-test p-values less than 0.01 and a fold change value of 2 were considered significantly differentially transcribed between the two samples compared.

5.2.4.3 Validation of candidate genes using qRT-PCR

The top candidate genes that were significantly differentially expressed from the microarray analysis were selected for qPCR validation. The materials and method used for the qPCR is described in Chapter 2. The total RNA from 3 replicates of samples PG, KL, JB and VCRU that was used in the microarray was used to synthesis the cDNA for the qRT-PCR. Permethrin resistant KL strain was also used in the qRT-PCR. In addition, expression of the genes was also investigated in the samples from KB that was not used in the microarray to access the potential role in this population. The primers used are listed in Table 5.1.

Primer Name	Sequence	Product size (base pairs)	SYBR Standards (RSq)	Efficiency (%)
qAlbCYP6N3 F	AAAATTGCATAAATGAAACTC TTCGTAAA	124	0.987	94.9
qAlbCYP6N3 R	ATCATCACCGACGTGCCTTT			
qAlbCYP9AE1 F	TTGGAATGACGACGAGTTGA	132	0.962	110.2
qAlbCYP9AE1 R	TCGAATAACCGCTCCTGAAT			
qAlbGSTT3 F	GAGGAAATTTGAAAACCGTTC GTC	133	0.999	92.2
qAlbGSTT3 R	GAACTCCCGCGACAGATACC			
qAlbCYP9J17 F	TGGATCGTTTGTGATCGAGA	148	0.994	103.6
qAlbCYP9J17 R	AGGCGTACGGATTGATCTTC			
qAlbABCA F	CTTTGGATTGTTAGGAATGAA CGGA	150	0.989	89.3
qAlbABCA R	TAACCGTACTGGGAGCGGTA			
qAlbCYP6M2 F	TCACACTGGAGAAGGACTGC	117	0.968	90.0
qAlbCYP6M2 R	CACTCTGGTCCGGATTGAAG			
qAlbGSTD1 F	GGGTCCAGTTGAACCTGAAG	144	0.995	98.6
qAlbGSTD1 R	TTTGAATGGCTCTGCTTTCC			
qAlbCYP6P12 F	CGTGCGCTTTTGGGATTGAG	145	0.996	94.7
qAlbCYP6P12 R	ATCGTCCGTGCCAAATCCTT			
qAlbSCD01845 F	GACCGCCAAGAATGGGAAGA	134	0.985	95.4
qAlbSCD01845 R	AGCAAACAAGCCTTCGAGGT			
qAlbCYP9M6 F	AGTTGGCAGCTACTGGAGGA	116	0.981	105.2
qAlbCYP9M6 R	GAAATCAGCTGCTTCCTTGG			
qAlbSCD15871 F	GAGGAACGTTCCTAGTATCCA AGG	110	0.953	105.8
qAlbSCD15871 R	GGCGAACCATTCCGGTTCTA			
qTub-Aae F	CCGCACTCGAGAAGGATTAC	131	0.998	102.0
qTub-Aae R	GTGGTTCGGTTTGACTTCGT			
qRPS7-Aae F	AAGGTCGACACCTTCACGTC	131	0.999	95.1
qRPS7-Aae R	CGCGCGCTCACTTATTAGAT			

Table 5.1 Primers used for qRT-PCR for microarray candidate genes validation in *Ae. albopictus*.

5.3 Results

5.3.1 Species Identification

The PCR based species identification (Sp. ID) method of Beebe *et al.* (2007) that was conducted to confirm the species of the samples showed that 100% of the samples that were previously identified by morphological differences as *Ae. albopictus* were the correct species (Table 4.3).

The gel electrophoresis images obtained after conducting the PCR showed the correct bands for identification of *Ae. albopictus* samples after digestion with *RsaI* with major bands at 190, 200 and 290bp as shown in chapter 4 (Figure 4.3 & 4.4).

5.3.2 Detection of *kdr* mutations in *Ae. albopictus* using pyrosequencing

Unfortunately after conducting preliminary test using the F_0 samples, the pyrosequencing method failed to show good results for the three possible mutations analysed [Exon 20 [I1011V (or M)] (Brengues *et al.*, 2003), Exon 21 [V1016I (or G)] (Saavedra-Rodriguez *et al.*, 2007) and Exon 31 [F1534] (Harris *et al.*, 2010)]. Primers used for *Ae. aegypti* matched well the sequence in *Ae. albopictus*, the alignment of the exons between the two species are shown in Figure 5.3.

PCR amplifications were successfully obtained for all three *kdr* mutations before running the samples on the pyrosequencer (Figure 5.4). However, results could not be obtained or all of the samples showed as 'failed' in the pyrograms. Change of the "sequence to analyse" of the respective pyrosequencing reactions in the pyrosequencer program for each of the three mutations also failed to improve the results.

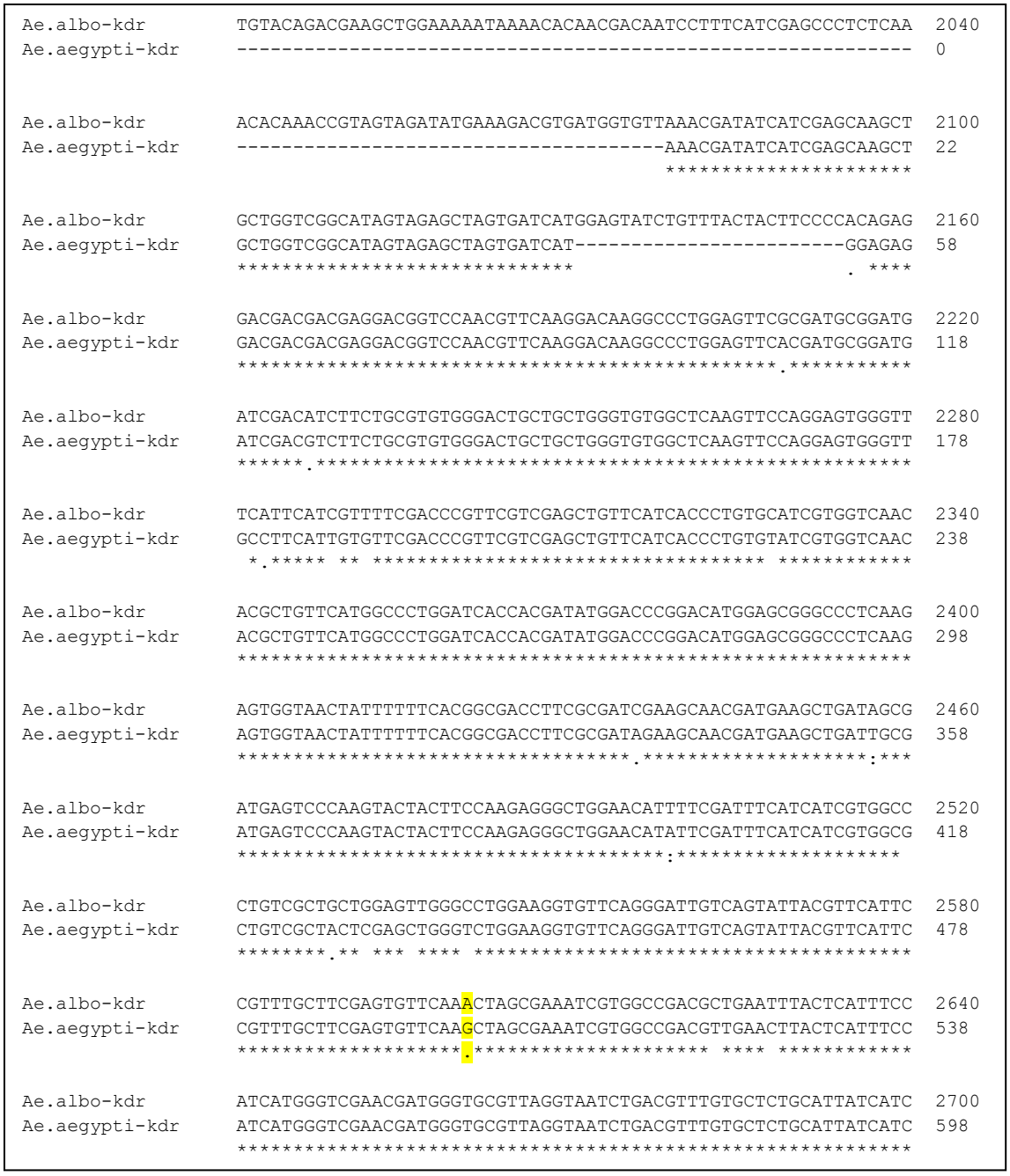

Figure 5.3 The alignment of *Ae. aegypti* and *Ae. albopictus* VGSC gene exons using ClustalW (Thompson et al., 1994). Highlighted yellow is the gene location of mutation.

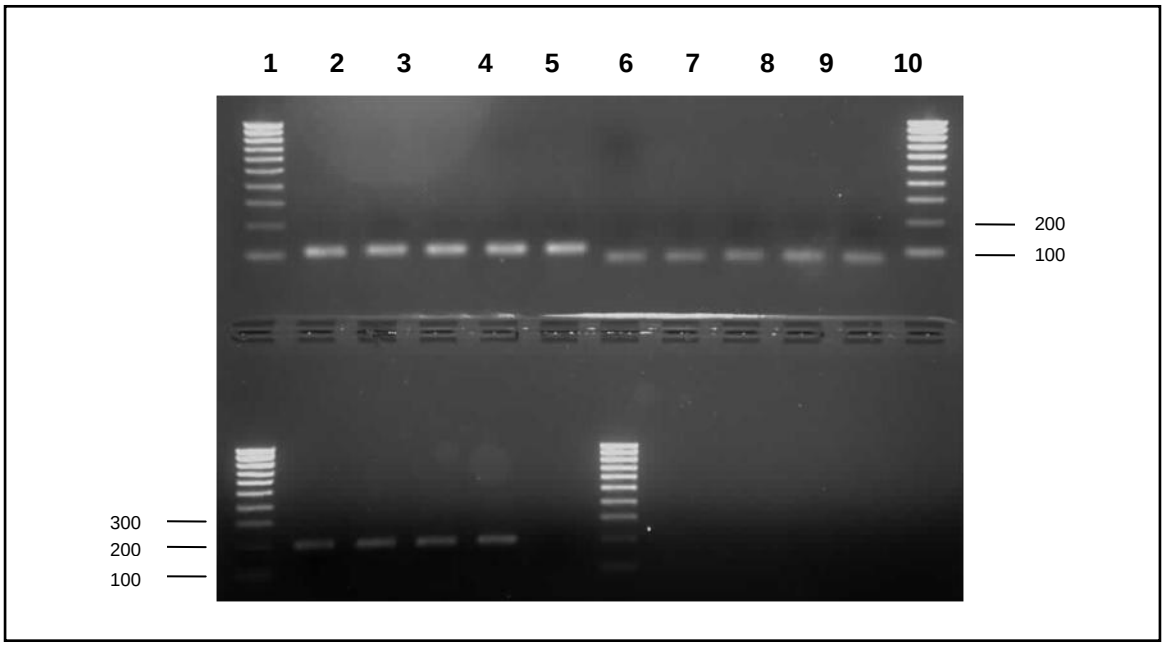

Figure 5.4 Gel electrophoresis image showing strong bands after pyrosequencing PCR amplification for three types of kdr mutations in *Aedes albopictus*.
(a) Lane 2 – 6: *kdr*1011, Lane 7 – 11: *kdr*1016 (b) Lane 2-5: *kdr*1534

5.3.3 Search for potential kdr mutations in *Ae. albopictus* in Malaysia

The presence of potential *kdr* mutations in *Ae. albopictus* in Malaysia was further investigated by sequencing the cDNA fragment spanning exons 19 to 31. The PCR amplification successfully generated a PCR product of 2586bp as expected for 12 tested cDNA samples all DDT resistant. Direct sequencing and alignment produced two fragments properly aligned for all samples. The first fragment (Frag-1) covered a size of 504bp from codon 919 to 1085 and was obtained with the forward primer. The second fragment (Frag-2), of a size of 1099bp resulted from the sequencing with the reverse primer and covered codons 1339 to 1704 of the gene. An indel from codon 1085 prevented the direct sequencing of the following sequence for all samples and therefore, codon 1086 to 1338 could not be covered in this study. Each of these two fragments was consequently analysed separately because not the same samples were successfully sequenced for both.

Polymorphism analysis: Frag-1 (n=18) exhibited 7 substitutions across Malaysia, all of which were at the third coding position. A nucleotide diversity (π) of $2.4x10^{3}$ was observed with a total of 8 haplotypes. Further information on the genetic parameters is indicated in Table 5.2 (n. pie, h (hd) Tajima D and D* frequency of predominant haplotypes). No amino acid change was recorded suggesting that the 1011 and the 1016 *kdr* mutations observed in *Ae. aegypti* are absent in *Ae. albopictus* in Malaysia (Figure 5.6).

A total of 13 substitutions were recorded for Frag-2, of which 11 were at the third coding position and 2 at the first coding position. A nucleotide diversity (π) of $3.4x10^{3}$ was observed with a total of 14 haplotypes. Again, no amino acid change was observed in these samples suggesting that the F1534C mutation observed in *Ae. aegypti* and reported recently in *Ae. albopictus* in Singapore (Kasai *et al.*, 2011) was absent in these populations (Figure 5.7). Further information on the genetic parameters of Frag-2 is indicated in Table 5.3.

5.3.3.1 Genetic diversity of the VGSC in Malaysia

Analysis of the Phylogenetic tree of the haplotypes for both fragments indicated that haplotypes are genetically diverse and do not cluster by location (Figure 5.5 and 5.6). The lack of a predominant haplotypes is an indication that the VGSC gene is not under selection pressure in Malaysian population of *Ae. albopictus* and support the absence of *kdr* mutation in this study.

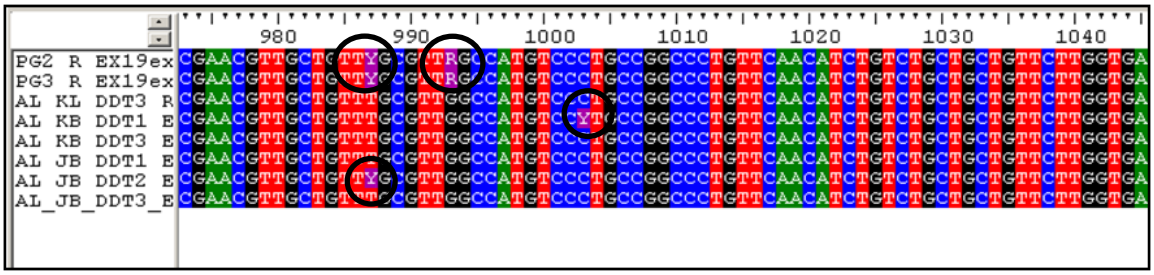

Figure 5.5 Sequence alignment showing presence of mutation (in circle).

Samples	N	S	h (hd)	Syn	NSyn	π (k)	D	D*
Frag-1								
Penang	6	3	4 (0.800)	3	0	0.00291 (1.47)	0.60031	0.51052
Kuala Lumpur	4	1	2 (0.667)	1	0	0.00132 (0.67)	1.63299	1.63299
Johor Bharu	6	2	3 (0.600)	2	0	0.00172 (0.87)	-0.05002	0.06221
Kota Bharu	2	1	2 (1.000)	1	0	0.00198 (1.00)	-	-
Combined	18	7	8 (0.699)	7	0	0.00249 (1.25)	-1.30098	-0.51292

Table 5.2 Summary statistics for polymorphism at fragment 1 (frag-1) of the voltage gated sodium channel (VGSC) gene spanning exon 19 to 31 in DDT resistant *Ae. albopictus* samples from four locations.

N= number of sequences (2n); S, number of polymorphic sites; h, Number of haplotypes (haplotype diversity); Syn, synonymous; Nsyn, non-synonymous; π, nucleotide diversity (k= mean number of nucleotide differences); Tajima's D and Fu and Li's D statistics.

Samples	N	S	h (hd)	Syn	NSyn	π (k)	D	D*
Frag-2								
Penang	4	4	3 (0.833)	4	0	0.00227 (2.50)	1.36522	1.36522
Kuala Lumpur	2	5	2 (1.000)	5	0	0.00455 (5.00)	-	-
Johor Bharu	6	9	6 (1.000)	9	0	0.00364 (4.00)	0.08824	-0.09221
Kota Bharu	4	6	4 (1.000)	6	0	0.00288 (3.17)	-0.31446	-0.31446
Combined	16	13	14 (0.983)	13	0	0.00342 (3.76)	-0.15693	-0.29043

Table 5.3 Summary statistics for polymorphism at fragment 2 (frag-2) of the voltage gated sodium channel (VGSC) gene spanning exon 19 to 31 in DDT resistant *Ae. albopictus* across all four populations.

N= number of sequences (2n); S, number of polymorphic sites; h, Number of haplotypes (haplotype diversity); Syn, synonymous; Nsyn, non-synonymous; π, nucleotide diversity (k= mean number of nucleotide differences); Tajima's D and Fu and Li's D statistics.

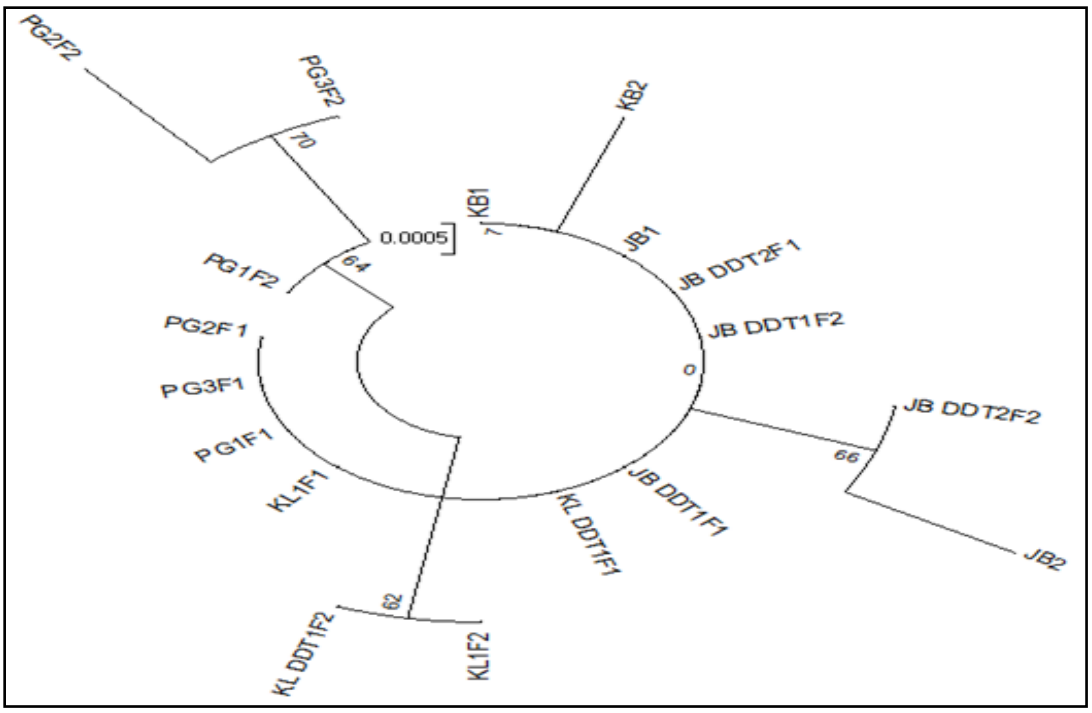

Figure 5.6 Phylogenetic tree of the haplotypes for Frag-1 constructed using TCS1.21 jar software.

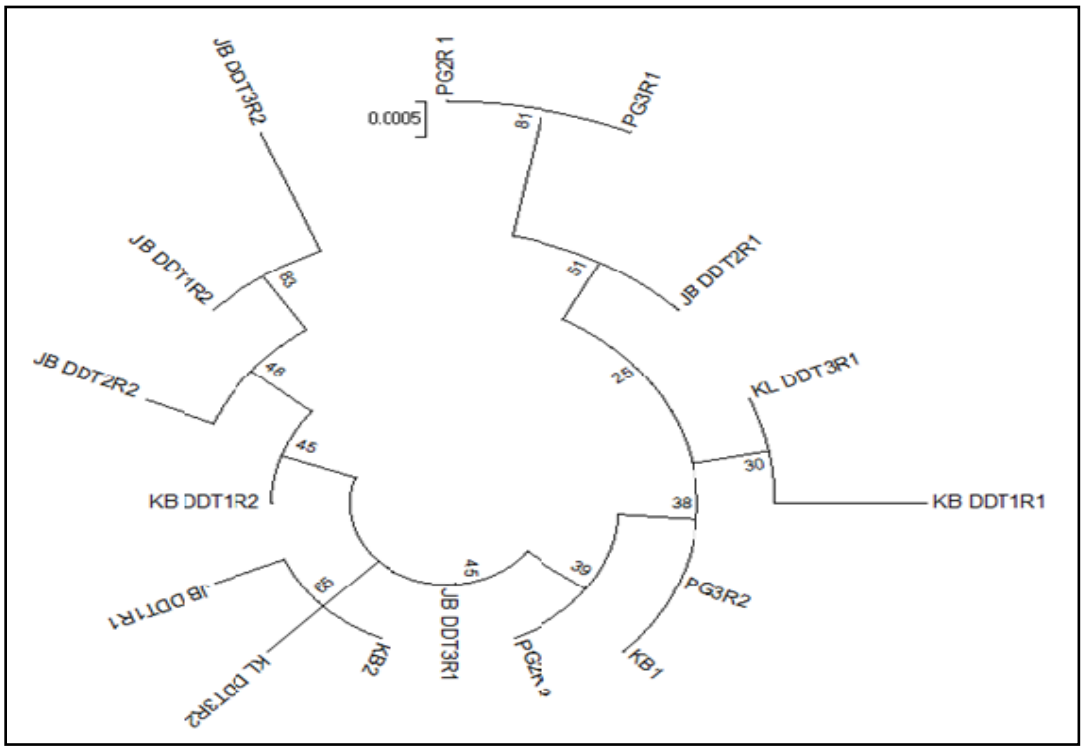

Figure 5.7 Phylogenetic tree of the haplotypes for Frag-2 constructed using TCS1.21 jar software.

5.3.4 Sequencing of *Ace-1* gene

The PCR amplification the Ace-1 gene successfully generated a PCR product of 2098 bp as expected for all the bendiocarb resistant cDNA samples (Figure 5.8). However, the presence of Ace-1 gene could not be determined. The data obtained after the samples were sent for sequencing and alignment was not of good quality in both forward and reverse directions. This was due to the presence of alternative splicing as pooled of cDNA were sequenced. There are double peaks when the data was observed using the Bioedit software which could lead to genotyping a mutation that is not really present. The best way is to clone the samples but unfortunately time was a constraint when conducting this research.

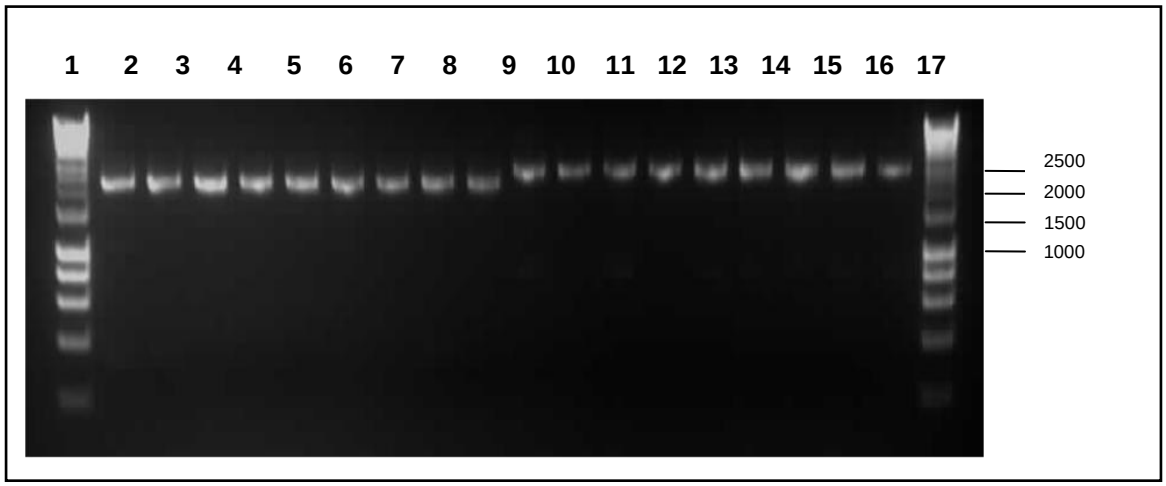

Figure 5.8 Gel electrophoresis image showing strong bands after *Ace-1* gene PCR amplification.
Lane 2-10: *Ae. albopictus*, Lane 11-19: *Ae. aegypti*

5.3.5 Genome-wide transcriptome profiling of *Ae. albopictus* populations in Malaysia using microarray

The microarrays were used to compare the genome-wide transcriptome between the susceptible VCRU laboratory strain, non-exposed field strains and permethrin resistant Kuala Lumpur strain. The two experimental designs of the microarray

are as described in Section 5.2.4.1 but the analysis is divided into three types of comparisons. The first is analysis is the comparison of non-exposed (control) field strain from KL, PG and JB against VCRU lab strain (C-S). The second analysis is comparing the non-exposed and permethrin resistant KL strains against VCRU lab strain (R-C, R-S and C-S). The final analysis is PG and JB (C-S) against KL (R-C).

The quality control (QC) analysis showed a good score with 11/11 and only 1 hybridization showed 10/11. Differentially expressed probes with fold change (FC) value ≥2 and p values of either 0.05 or 0.01 was used. Data sets were then recorded in the form of Venn diagram as shown in Figure 5.8, 5.9 and 5.10.

5.3.5.1 Comparison of control strains from PG, KL and JB against susceptible VCRU lab strain

The first comparison carried out using *Ae. albopictus* samples were C-S samples from PG, KL and JB. From the analysis conducted, PG samples showed the highest number of deferentially expressed probes with 4443 probes followed by Johor Bharu and Kuala Lumpur with 1332 and 488 probes respectively (Figure 5.9). The numbers of commonly transcribed probes were 100 with 40 probes up-regulated and 60 probes down-regulated.

Genes commonly up-regulated in the three locations

To identify potential resistance candidate genes, the probes from the commonly differentially expressed probes were further analysed. Firstly, more attention was paid on the commonly up-regulated probes between the three locations since this is a strong indication of possible role in the common resistance observed in the three locations particularly for bendiocarb resistance which is the common resistance (Table 3.7). Among the 40 up-regulated probes, genes from various gene families were observed. The detoxification genes included only three cytochrome P450s. Other probes up-regulated belong to other gene families

such as redox, protein synthesis, ion transport, immune defence and others (Table 5.4). Overall the most over-expressed gene was Holotricin which is an antimicrobial peptide belonging to immune defence family.

Out of the three cytochrome P450 genes commonly up-regulated, the P450 gene from the HQ621851 transcript, which is the closest hit (in NCBI) to CYP6N9 in *Ae. aegypti* was the most up-regulated in PG with FC of 11.067 while it had a comparable expression in JB and KL with respective FC of 3.3907 and 3.8054. The second P450 gene from the transcript, Aalb_oocyte_rep_c24780, with the closest hit in NCBI was CYP9AE1 in *Ae. aegypti*, showed a higher FC value of 6.3497 in PG followed by JB with 4.0064 and KL with 2.1861. The final detoxification gene that was up-regulated was obtained from a probe designed from the *Ae. aegypti* CYP6AG6 P450 gene (AAEL006992-RA). For this gene, KL showed the most up-regulation with FC of 9.6016 and followed by PG with 3.0140 and JB with 2.7691.

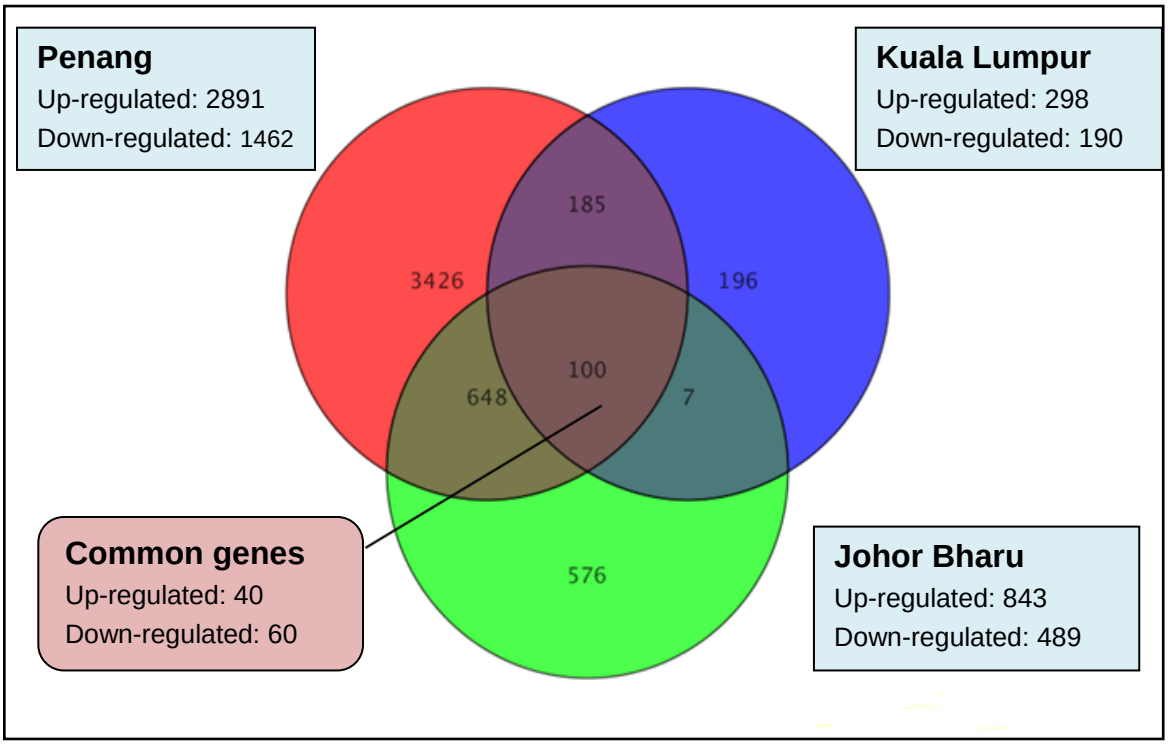

Figure 5.9 Venn diagram of differentially transcribed genes in *Ae. albopictus* for KL, PG and JB C-S (p= 0.05)

Genes commonly up-regulated in KL and PG but not in JB

Because the susceptibility profiles were different between the three locations, it was most likely that the expression profiles could vary. To assess this variation, sets of genes commonly over-expressed only between two locations were analysed particularly in relation to the known susceptibility profile. The set of detoxification genes commonly over-expressed in KL and PG only includes 3 cytochrome P450s. The most over-expressed of these P450s is CYP6N3 for which three probes belonging to 3 different transcripts of the gene (Genbank) are consistently over-expressed in the same range of FC. A higher expression is consistently observed in PG than in KL with FC ranging from 22 to 25 in PG and 7.4 to 7.8 in KL. Another P450 that was over-expressed with similar level of expression in the two locations came from the *Ae. albopictus* Aalb_oocyte_rep_c28874 transcript. The closest hit for this transcript corresponds to the P450 CYP6AG6 in *Ae. aegypti*. The last P450 was obtained with the probes from *Ae. aegypti* transcript (AAEL009656-RA) corresponding to CYP6AL3. Other over-expressed genes from this list belong to Alcohol dehydrogenase, hydrolase, juvenile hormone and others (Table 5.5).

Genes commonly up-regulated in PG and JB but not in KL

More genes were commonly over-expressed in PG and JB than between PG and KL. From the 34 number of detoxification probes over-expressed, nearly half belongs to P450 genes (20), 5 to GSTs, 4 to ABC transporters and 1 carboxylesterase with some proteases also observed. Among the cytochrome P450s, most over-expressed genes belong to the CYP6 family and the remaining to the CYP9 family. The CYP6 genes over-expressed include CYP6N3 (4 probes), a P450 transcript with the closest hit to CYP6P4 in *An. gambiae* (5 probes) and another P450 transcript with the closest hit to CYP6P12 in *Ae. aegypti* (5 probes). Additionally, 1 probe was over-expressed for transcripts with the closest hit to CYP6Z6, CYP6Z8 and CYYP6ZB1 in *Ae. aegypti*. The two CYP9 genes were transcripts with the closest hit to CYP9AE1 and CYP9M6 in

Ae. aegypti. From this list, the most over-expressed P450 genes in PG was CYP9AE1 (FC 6.68) while CYP6P12 was the most over-expressed in JB (FC 10.52) (Table 5.6).

The 5 over-expressed GST probes belong to three genes with the closest hit to the *Ae. aegypti* GSTT3 (1 probe), GSTD5 (2 probes) and GSTE3 (2 probes). A consistent higher over expression of all the GSTs was observed in JB than PG. The top up-regulated GST in JB was GSTD5 with FC value of 13.73 but an FC of 4.36 in PG (Table 5.3). The unique carboxylesterase gene showed a similarly low expression in both PG (FC 2.44) and JB (FC 2.34) (Table 5.6).

Table 5.4 Probes commonly up-regulated in *Ae. albopictus* in all three locations in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute – FC			Description
		Penang	Johor Bharu	Kuala Lumpur	
Detoxification					
CUST_90_PI427639958	HQ621851.1 (as CYP6N9 in *Ae. aegypti*)	11.06717	3.390716	3.80548	Cytochrome P450
CUST_17858_PI427639955	Aalb_oocyte_rep_c24780 (as CYP9AE1 in *Ae. aegypti*)	6.349737	4.006419	2.186192	Cytochrome p450
CUST_15781_PI427639947	AAEL006992-RA (as CYP6AG6 in *Ae. aegypti* & CYP6AG1 in *An. gambiae*)	3.014005	2.769197	9.60161	Cytochrome p450
Redox/mitochondrial					
CUST_18659_PI427639955	Aalb_oocyte_rep_c13574	3.46779	7.934232	3.256955	acetyl- mitochondrial
Protein synthesis/metabolism					
CUST_33850_PI427639947	AAEL014562-RA	3.267709	3.464005	2.240465	60S ribosomal protein L12
CUST_3111_PI427639955	Aalb_oocyte_rep_c35888	5.467519	6.689628	2.321785	cathepsin l
CUST_24197_PI427639947	AAEL008853-RA	3.431536	3.63372	2.979337	choline/ethanolamine kinase
CUST_9602_PI427639955	Aalb_oocyte_rep_c42656	3.936562	3.891619	2.101489	mitochondrial ribosomal protein l44
CUST_5613_PI427639955	Aalb_oocyte_rep_c21876	6.808945	10.22998	5.400242	probable ribosome biogenesis protein c16orf42-like
CUST_13353_PI427639955	Aalb_oocyte_GIK0NFC01AZEAV	8.486282	6.111363	2.1815	serine protease
CUST_12040_PI427639955	Aalb_oocyte_rep_c61861	4.325947	3.670383	2.0646	serine protease
CUST_20414_PI427639955	Aalb_oocyte_rep_c2630	3.255203	2.629312	2.35589	serine threonine-protein kinase rio2
Lipid/carbohydrate synthesis/metabolism					
CUST_2982_PI427639955	Aalb_oocyte_GH79BIP02FWVVV	4.160906	4.088471	2.971394	lipoprotein lipase
Transport/ion transport					
CUST_13058_PI427639947	AAEL008381-RA	2.248142	3.224599	2.168007	oligopeptide transporter
CUST_5895_PI427639955	Aalb_oocyte_rep_c7850	3.644206	2.972166	2.548524	sugar transporter
Immune defence					
CUST_30823_PI427639947	AAEL017536-RA	12.97018	12.63358	2.931466	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide.
CUST_763_PI427639955	Aalb_oocyte_rep_c27383	10.03429	6.767646	2.126202	hypothetical mtt-rich mucin
Other					
CUST_15452_PI427639947	AAEL009396-RA	2.8108	3.307311	2.689146	amine oxidase

CUST_11965_PI427639955	Aalb_oocyte_rep_c10601	12.65986	28.7097	3.635909	t-dirnahydrouridine synthase
CUST_21247_PI427639947	AAEL007160-RA	12.22205	12.42675	9.207074	ubiquilin 1,2
CUST_17631_PI427639955	Aalb_oocyte_GH79BIP02GTORD	6.993401	8.338801	5.054333	AGAP006143-PE [Anopheles gambiae str. PEST]
CUST_12982_PI427639955	Aalb_oocyte_rep_c61338	6.629262	4.511744	2.134098	cg31751 cg31751-pa
CUST_6496_PI427639955	Aalb_oocyte_rep_c1566	3.750916	4.260312	2.801973	cg4553 cg4553-pa
CUST_32485_PI427639947	AAEL014556-RB	2.942438	3.009454	2.273961	conserved hypothetical protein
CUST_29183_PI427639947	AAEL015053-RB	2.808157	3.227621	2.324586	conserved hypothetical protein
CUST_19802_PI427639947	AAEL006433-RA	2.561922	3.224315	2.347336	conserved hypothetical protein
CUST_7711_PI427639947	AAEL003929-RA	2.55509	8.356735	3.328963	conserved hypothetical protein
CUST_1287_PI427639947	AAEL000615-RA	2.011041	5.307753	2.189566	hypothetical protein
CUST_20050_PI427639955	Aalb_oocyte_GH79BIP01CBET4	5.582831	3.567648	2.654278	hypothetical protein AaeL_AAEL013040
CUST_3055_PI427639955	Aalb_oocyte_c20316	8.572461	2.958011	6.382831	kda secreted protein -1
CUST_4527_PI427639955	Aalb_oocyte_rep_c58107	2.195233	2.221553	2.024178	kda secreted salivary peptide

Table 5.5 Probes from detoxification genes & genes linked with resistance commonly up-regulated in *Ae. albopictus* in PG and KL but not JB in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute – FC		Description
		Penang	Kuala Lumpur	
CUST_115_PI427639958	JF317342.1 (CYP6N3)	25.10652	7.812811	Aedes albopictus clone EV2 cytochrome P450 mRNA, complete cds
CUST_118_PI427639958	JF317340.1 (CYP6N3)	23.14278	7.880488	Aedes albopictus clone EV4 cytochrome P450 mRNA, complete cds
CUST_130_PI427639958	JF317341.1 (CYP6N3)	22.66409	7.46686	Aedes albopictus clone EV3 cytochrome P450 mRNA
CUST_12986_PI427639947	AAEL011130-RA	5.79561	8.328121	alcohol dehydrogenase
CUST_20950_PI427639955	Aalb_oocyte_rep_c32791	4.489691	2.963726	juvenile hormone epoxide hydrolase 1
CUST_2455_PI427639955	Aalb_oocyte_c28776	4.366414	2.610582	serine protease 14
CUST_6168_PI427639947	AAEL002133-RA	3.025169	2.140621	juvenile hormone-inducible protein, putative
CUST_18637_PI427639955	Aalb_oocyte_rep_c28874 (as CYP6AG6 in *Ae. aegypti*)	2.915741	2.847051	cytochrome p450
CUST_20670_PI427639955	Aalb_oocyte_rep_c55379	2.095733	2.429616	oxidative stress-induced growth inhibitor 1-like
CUST_13399_PI427639947	AAEL009656-RA (CYP6AL3 in *Ae. aegypti*)	2.045239	2.880639	cytochrome P450

Table 5.6 Probes from detoxification genes & genes linked with resistance commonly up-regulated in *Ae. albopictus* PG and JB but not KL in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute – FC		Description
		Penang	Johor Bharu	
CUST_17859_PI427639955	Aalb_oocyte_rep_c24780 (as CYP9AE1 in *Ae. aegypti* and CYP9E2 in Blatella germanica)	6.6812	4.8599	cytochrome p450
CUST_35612_PI427639947	AAEL015432-RA	6.965575	3.475481	trypsin, putative
CUST_1663_PI427639955	Aalb_oocyte_rep_c11155 (GSTT3 in *Ae. aegypti*)	6.491828	10.19155	glutathione-s-transferase gst
CUST_19417_PI427639955	Aalb_oocyte_rep_c4101	5.602456	7.361225	abc transporter
CUST_19418_PI427639955	Aalb_oocyte_rep_c4101	5.399948	11.0266	abc transporter
CUST_263_PI427639958	AF284783.1 (CYP6N3)	5.25585	5.592339	Aedes albopictus cytochrome P450 CYP6N3v4 mRNA, partial cds
CUST_21111_PI427639955	Aalb_oocyte_GH79BIP02HN8AL	4.997693	4.606106	atp-binding cassette transporter
CUST_7878_PI427639947	AAEL005491-RA	4.629775	5.31701	ABC transporter
CUST_21101_PI427639955	Aalb_oocyte_c13494 (as CYP9M6 in *Ae. aegypti* and CYP9M2 as in gambiae)	4.628993	6.076844	cytochrome p450
CUST_12041_PI427639955	Aalb_oocyte_rep_c61861	4.492053	3.750054	serine protease
CUST_21328_PI427639955	Aalb_oocyte_c30071 (as GSTD5 in *Ae. aegypti*)	4.363947	13.73232	glutathione s-transferase
CUST_21329_PI427639955	Aalb_oocyte_c30071 (as GSTD5 in *Ae. aegypti*)	3.764468	8.88213	glutathione s-transferase
CUST_9941_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	3.453267	10.51562	cytochrome p450
CUST_9942_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	3.299378	7.390336	cytochrome p450
CUST_92_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	3.293268	3.742736	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_93_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	3.072198	3.764997	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_32014_PI427639947	AAEL013936-RC	2.920809	3.438896	serine protease inhibitor (serpin), likely cleavage at I/S. Transcript A.
CUST_857_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in	2.800269	4.632875	cytochrome p450

	Ae. aegypti and CYP6P4 in *An. gambiae*)			
CUST_91_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	2.794235	3.133532	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_87_PI427639958	HQ621853.1 (CYP6N3)	2.632891	2.14199	Aedes albopictus isolate P16 cytochrome P450 mRNA, partial cds
CUST_86_PI427639958	HQ621853.1 (CYP6N3)	2.587828	2.280029	Aedes albopictus isolate P16 cytochrome P450 mRNA, partial cds
CUST_85_PI427639958	HQ621853.1 (CYP6N3)	2.55623	2.243699	Aedes albopictus isolate P16 cytochrome P450 mRNA, partial cds
CUST_981_PI427639955	Aalb_oocyte_rep_c46923 (as GSTE3 in *Ae.aegypti*)	2.555421	3.858838	glutathione-s-transferase gst
CUST_135_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	2.537406	3.029287	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_9720_PI427639955	Aalb_oocyte_GIK0NFC01CWBYU	2.44061	2.344885	carboxylesterase
CUST_21999_PI427639955	Aalb_oocyte_rep_c13281 (as CYP6Z8 in *Ae. aegypti*)	2.390325	3.935278	cytochrome p450
CUST_9506_PI427639955	Aalb_oocyte_rep_c6282	2.331533	2.228077	atp-binding cassette sub-family a member
CUST_982_PI427639955	Aalb_oocyte_rep_c46923 (as GSTE3 in *Ae.aegypti*)	2.326212	5.305324	glutathione-s-transferase gst
CUST_22316_PI427639947	AAEL009123-RA (CYP6Z6 in *Ae.aegypti*)	2.281195	4.660739	cytochrome P450
CUST_134_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	2.278028	2.729131	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_858_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 in *An. gambiae*)	2.271363	4.870811	cytochrome p450
CUST_122_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	2.048554	2.060227	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds
CUST_121_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	2.030469	2.023919	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds
CUST_123_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	2.019654	2.012997	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds

Genes commonly up-regulated in KL and JB but not in PG

For analysis of data that were commonly over-expressed in KL and JB, there were no common differentially expressed probes that could be observed at the p value of 0.05.

Genes up-regulated in PG only

The number of differentially expressed probes that were present uniquely in PG was 3426 at $P<0.05$. Out of the 3426 probes the top up-regulated probes from detoxification genes and genes linked to resistance consist of a mixture of cytochrome P450s, GST, ABC transporters, cuticle proteins with also several proteases detected (Table 5.7).

The most up-regulated detoxification gene in PG, with FC value of 9.63, was a GST gene from the Aalb_oocyte_rep_c11155 transcript with the closest hit to the GSTT3 gene in *Ae. aegypti* after blasting in NCBI. However, a different probe for the same gene was also among the commonly up-regulated in the PG and JB comparison (Table 5.7).

Among the most over-expressed cytochrome P450s, the CYP9J17 gene which is the closest hit in *Ae. aegypti* (Aalb_oocyte_rep_c11991 transcript) could be observed. This gene is uniquely and consistently over-expressed in PG with FC values of 6.03 and 5.66 for its two probes. Another cytochrome P450 that could be observed only in PG was from the *Ae. aegypti* AAEL015370-RA transcript which was the closest hit to the CYP4J9 gene in *An. gambiae* (FC 5.46). This gene is not named for *Ae. aegypti* from vectorbase. Three other cytochrome P450 genes that were over-expressed in PG with transcripts with the closest hit to *Ae. aegypti* in NCBI were CYP6N11 (Aalb_oocyte_c25640), CYP6N12 (AAEL009124-RA) and CYP6J16 (AAEL014019-RA). Other P450s present were CYP6N3 and CYP6P12 (Table 5.7).

Genes up-regulated in JB only

Out of the 576 differentially expressed probes uniquely in JB, there were 22 up-regulated detoxification gene probes among them including cytochrome P405s, GSTs, ABC transporters, trypsin, oxidases, hydrogenases and others. The most up-regulated detoxification probe in JB was from an *Ae. aegypti* transcript (AAEL001061-RB) from GSTD1 gene . The two probes of this gene from the same transcript were consistently up-regulated with FC value of 4.59 and 4.22. Three different cytochrome P450 transcripts were up-regulated uniquely in JB. CYP9J27 and CYP9J15 in *Ae. aegypti* were the closest hits respectively to Aalb_oocyte_rep_c925 and Aalb_oocyte_rep_c15442 transcripts whereas transcript AF284786.1 corresponds to CYP6N4 in *Ae. albopictus*. Overall, these uniquely up-regulated P450s in JB had lower expression levels with 2.23 FC for CYP9J27, 2.13 for CYP9J15 and 3.76 for CYP6N4. This list also included unique probes corresponding to genes also expressed in other locations such as for CYP6Z8 and CYP6ZB. An aldehyde oxidase gene was also up-regulated in JB with an FC value of 3.66 (Table 5.8).

Genes up-regulated in KL only

In KL, the number of differentially expressed probes was low compared to other locations with only 196 probes (Figure 5.7).Out of the 196 probes, only 4 cytochrome P450s genes and 1 GST genes were uniquely up-regulated in KL. Other potential resistance genes up-regulated are listed in Table 5.9.

The CYP6AK1 gene from the *Ae aegypti* AAEL004941-RA transcript was the most up-regulated amongst the cytochrome P450s (FC 2.89). Another cytochrome P450 was the CYP6AG5 gene with the closest hit to *Ae. aegypti* in NCBI (transcript Aalb_oocyte_GH79BIP02I9E16). Both probes corresponding to this gene was consistently up-regulated with FC values of 2.53 and 2.33. CYP6AG6 (transcript Aalb_oocyte_rep_c28874) was another *Ae. aegypti* cytochrome P450 that was over-expressed in JB with an FC value of 2.26. Two

probes corresponding to GSTD1 gene of the *Ae. albopictus* Aalb_oocyte_rep_c8445 transcript was also consistently up-regulated in KL with FC values of 2.087 and 2.086 (Table 5.9).

Genes commonly down-regulated in the three locations

Out of the top genes commonly down-regulated in PG, JB and KL, vitellogenin and vitelline protein seems to be consistently down-regulated. Probes with unknown function were amongst the top commonly down-regulated. No detoxification genes could be observed in the commonly down-regulated genes (Table 5.10).

Table 5.7 Top 30 probes from detoxification genes & genes linked with resistance up-regulated in *Ae. albopictus* in PG only not in KL and JB in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute - FC	Description
CUST_1664_PI427639955	Aalb_oocyte_rep_c11155 (as GSTT3 in *Ae. aegypti*)	9.627461	glutathione-s-transferase gst theta
CUST_2907_PI427639947	AAEL001101-RA	6.780925	ATP-dependent transporter
CUST_132_PI427639958	JF317341.1 (CYP6N3)	6.638058	Aedes albopictus clone EV3 cytochrome P450 mRNA, complete cds
CUST_2672_PI427639955	Aalb_oocyte_rep_c18373	6.03978	pupal cuticle
CUST_15444_PI427639955	Aalb_oocyte_rep_c11991 (as CYP9J17 in *Ae. aegypti*)	6.030248	cytochrome p450
CUST_2671_PI427639955	Aalb_oocyte_rep_c18373	5.985068	pupal cuticle
CUST_120_PI427639958	JF317340.1 (CYP6N3)	5.963319	Aedes albopictus clone EV4 cytochrome P450 mRNA, complete cds
CUST_15445_PI427639955	Aalb_oocyte_rep_c11991 (as CYP9J17 in *Ae. aegypti*)	5.661962	cytochrome p450
CUST_27663_PI427639947	AAEL015370-RA (as CYP4J9 in *An. gambiae*)	5.457839	cytochrome P450
CUST_2908_PI427639947	AAEL001101-RA	5.303114	ATP-dependent transporter
CUST_14077_PI427639955	Aalb_oocyte_rep_c24593	5.302811	serine protease
CUST_18993_PI427639955	Aalb_oocyte_rep_c21975	4.99746	serine protease
CUST_7355_PI427639955	Aalb_oocyte_c25640 (as CYP6N11 in *Ae. aegypti*)	4.964203	cytochrome p450
CUST_23124_PI427639947	AAEL009124-RA (CYP6N12 in *Ae. aegypti*)	4.957031	cytochrome P450
CUST_18994_PI427639955	Aalb_oocyte_rep_c21975	4.884067	serine protease
CUST_27662_PI427639947	AAEL015370-RA (as CYP4J9 in *An. gambiae*)	4.64563	cytochrome P450
CUST_8701_PI427639955	Aalb_oocyte_GH79BIP01EAIKO	4.399652	serine protease
CUST_7354_PI427639955	Aalb_oocyte_c25640 (as CYP6N11 in *Ae. aegypti*)	4.309329	cytochrome p450
CUST_8702_PI427639955	Aalb_oocyte_GH79BIP01EAIKO	4.201814	serine protease
CUST_14076_PI427639955	Aalb_oocyte_rep_c24593	4.097683	serine protease
CUST_27549_PI427639947	AAEL014019-RA (CYP4J16 in *Ae. aegypti*)	3.949462	cytochrome P450
CUST_14124_PI427639955	Aalb_oocyte_rep_c10261	3.8534	serine protease
CUST_17684_PI427639947	AAEL006586-RA	3.851256	serine protease

CUST_16173_PI427639947	AAEL006260-RA	3.586701	serine protease, putative
CUST_21973_PI427639955	Aalb_oocyte_rep_c21808 (as CYP6P12 in *Ae. aegypti*)	3.56301	cytochrome p450
CUST_35854_PI427639947	AAEL013834-RA	3.52104	ATP-binding cassette transporter
CUST_21250_PI427639947	AAEL006600-RA	3.508982	juvenile hormone-inducible protein, putative
CUST_3080_PI427639955	Aalb_oocyte_GH79BIP01ATT64	3.464188	juvenile hormone esterase
CUST_4198_PI427639947	AAEL002600-RA	3.404804	serine protease
CUST_10456_PI427639955	Aalb_oocyte_GH79BIP01BA1R3	3.31179	juvenile hormone esterase

Table 5.8 Probes from detoxification genes & genes linked with resistance up-regulated in *Ae. albopictus* in JB only not in KL and PG in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute - FC	Description
CUST_3159_PI427639947	AAEL001061-RB (GSTD1 in *Ae. aegypti*)	4.592515	glutathionetransferase
CUST_3160_PI427639947	AAEL001061-RB (GSTD1 in *Ae. aegypti*)	4.222839	glutathionetransferase
CUST_242_PI427639958	AF284788.1 (CYP6N4)	3.97193	Aedes albopictus cytochrome P450 CYP6N4v6 mRNA, partial cds
CUST_26373_PI427639947	AAEL014614-RA (as CYP9J4 in *An. gambiae*)	3.969994	cytochrome P450
CUST_245_PI427639958	AF284786.1 (CYP6N4)	3.759987	Aedes albopictus cytochrome P450 CYP6N4v4 mRNA, partial cds
CUST_27548_PI427639947	AAEL014019-RA (CYP4J16 in *Ae. aegypti*)	3.704473	cytochrome P450
CUST_21998_PI427639955	Aalb_oocyte_rep_c13281 (as CYP6Z8 in *Ae. aegypti*)	3.687491	cytochrome p450
CUST_22681_PI427639955	Aalb_oocyte_rep_c32138	3.659781	aldehyde oxidase
CUST_15200_PI427639955	Aalb_oocyte_rep_c7225	3.434868	short-chain dehydrogenase
CUST_8072_PI427639955	Aalb_oocyte_GIK0NFC01EFN86 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 *An. gambiae*)	2.828766	cytochrome p450
CUST_8073_PI427639955	Aalb_oocyte_GIK0NFC01EFN86 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 *An. gambiae*)	2.710069	cytochrome p450
CUST_7991_PI427639955	Aalb_oocyte_rep_c11431	2.537094	abc transporter
CUST_9872_PI427639955	Aalb_oocyte_GIK0NFC01AJOWD	2.399192	cuticular protein analogous to peritrophins 1-b
CUST_10104_PI427639955	Aalb_oocyte_rep_c29453	2.26926	juvenile hormone epoxide hydrolase 1
CUST_22585_PI427639955	Aalb_oocyte_rep_c925 (as CYP9J27 in *Ae.aegypti*)	2.235838	cytochrome p450
CUST_22584_PI427639955	Aalb_oocyte_rep_c925 (as CYP9J27 in *Ae.aegypti*)	2.1974	cytochrome p450
CUST_2000_PI427639955	Aalb_oocyte_rep_c15442 (as CYP9J15 in *Ae.aegypti*)	2.131997	cytochrome p450
CUST_11169_PI427639955	Aalb_oocyte_rep_c14697	2.083752	voltage-dependent para-like sodium channel
CUST_17303_PI427639955	Aalb_oocyte_rep_c34575	2.072003	heat shock protein 60
CUST_18374_PI427639955	Aalb_oocyte_rep_c61320	2.002443	short-chain dehydrogenase
CUST_5173_PI427639955	Aalb_oocyte_GH79BIP01BBCTN	2.002377	trypsin

CUST_32539_PI427639947	AAEL012189-RA	2.001069	multidrug resistance protein 1 (ATP-binding cassette C1)

Table 5.9 Probes from detoxification genes & genes linked with resistance up-regulated in *Ae. albopictus* in KL only not in PG and JB in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute - FC	Description
CUST_7183_PI427639955	Aalb_oocyte_GH79BIP02GCAQF	5.823614	juvenile hormone-inducible
CUST_7182_PI427639955	Aalb_oocyte_GH79BIP02GCAQF	5.818349	juvenile hormone-inducible
CUST_10095_PI427639947	AAEL004941-RA (CYP6AK1 in *Ae. aegypti*)	2.893056	cytochrome P450
CUST_22169_PI427639955	Aalb_oocyte_GH79BIP02I9E16 (as CYP6AG5 in *Ae. aegypti*)	2.53426	cytochrome p450
CUST_22168_PI427639955	Aalb_oocyte_GH79BIP02I9E16 (as CYP6AG5 in *Ae. aegypti*)	2.333974	cytochrome p450
CUST_10966_PI427639955	Aalb_oocyte_rep_c403	2.311656	chymotrypsin-like protein
CUST_10965_PI427639955	Aalb_oocyte_rep_c403	2.295476	chymotrypsin-like protein
CUST_18636_PI427639955	Aalb_oocyte_rep_c28874 (as CYP6AG6 in *Ae. aegypti*)	2.262525	cytochrome p450
CUST_22536_PI427639955	Aalb_oocyte_c27220	2.238019	juvenile hormone-inducible
CUST_21988_PI427639955	Aalb_oocyte_rep_c8445 (GSTD1)	2.087324	glutathione s-transferase d1
CUST_21989_PI427639955	Aalb_oocyte_rep_c8445 (GSTD1)	2.086734	glutathione s-transferase d1
CUST_22537_PI427639955	Aalb_oocyte_c27220	2.043962	juvenile hormone-inducible
CUST_5366_PI427639955	Aalb_oocyte_rep_c9368	2.041478	trna guanylyltransferase
CUST_7021_PI427639955	Aalb_oocyte_GH79BIP02JQ8K1 (as CYP6N17 in *Ae. aegypti*)	2.033709	cytochrome p450

Table 5.10 Top 20 commonly down-regulated probes in *Ae. albopictus* in all three locations in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute – FC			Description
		Penang	Johor Bharu	Kuala Lumpur	
CUST_22873_PI427639955	Aalb_oocyte_c33725	143.4072	26.43711	47.95689	vitellogenin-a1
CUST_22872_PI427639955	Aalb_oocyte_c33725	84.07549	16.65077	38.73036	vitellogenin-a1
CUST_7438_PI427639955	Aalb_oocyte_rep_c39281	59.03633	55.6601	19.48012	vitelline membrane protein homolog
CUST_18648_PI427639955	Aalb_oocyte_rep_c61804	57.7055	56.69963	13.24433	vitelline membrane protein homolog
CUST_18649_PI427639955	Aalb_oocyte_rep_c61804	57.11062	59.63628	13.25408	vitelline membrane protein homolog
CUST_22229_PI427639955	Aalb_oocyte_rep_c13033	47.36172	31.76793	20.74988	cathepsin b
CUST_20753_PI427639955	Aalb_oocyte_rep_c16648	45.61193	53.67629	14.8957	---NA---
CUST_22228_PI427639955	Aalb_oocyte_rep_c13033	39.76406	19.30259	17.3531	cathepsin b
CUST_20752_PI427639955	Aalb_oocyte_rep_c16648	38.90469	42.21267	13.77715	---NA---
CUST_6501_PI427639955	Aalb_oocyte_rep_c46407	36.42293	35.75332	8.109998	vitelline membrane protein homolog
CUST_6500_PI427639955	Aalb_oocyte_rep_c46407	35.10644	32.26815	8.037392	vitelline membrane protein homolog
CUST_21152_PI427639955	Aalb_oocyte_rep_c7314	30.77706	29.02226	12.25498	vitellogenin-a1
CUST_48_PI427639955	Aalb_oocyte_rep_c14495	29.54215	74.03451	6.373849	serine threonine-protein kinase rio2
CUST_23818_PI427639947	AAEL010434-RA	25.78022	20.00934	10.60109	Vitellogenin-A1 Precursor (VG)(PVG1)
CUST_28769_PI427639947	AAEL017403-RA	22.96659	24.13048	7.490641	Vitelline membrane protein 15a-2 Precursor
CUST_28768_PI427639947	AAEL017403-RA	22.84544	22.85535	7.476134	Vitelline membrane protein 15a-2 Precursor
CUST_22248_PI427639947	AAEL006670-RA	19.13723	21.27431	5.553213	conserved hypothetical protein
CUST_22247_PI427639947	AAEL006670-RA	16.52921	17.41917	7.153462	conserved hypothetical protein
CUST_7439_PI427639955	Aalb_oocyte_rep_c39281	16.27276	18.47254	13.06926	vitelline membrane protein homolog
CUST_16494_PI427639955	Aalb_oocyte_rep_c47052	11.6313	11.54597	6.185045	vitelline membrane protein homolog

5.3.5.2 Detection of specific pyrethroid resistance genes *in Aedes albopictus* (in Kuala Lumpur)

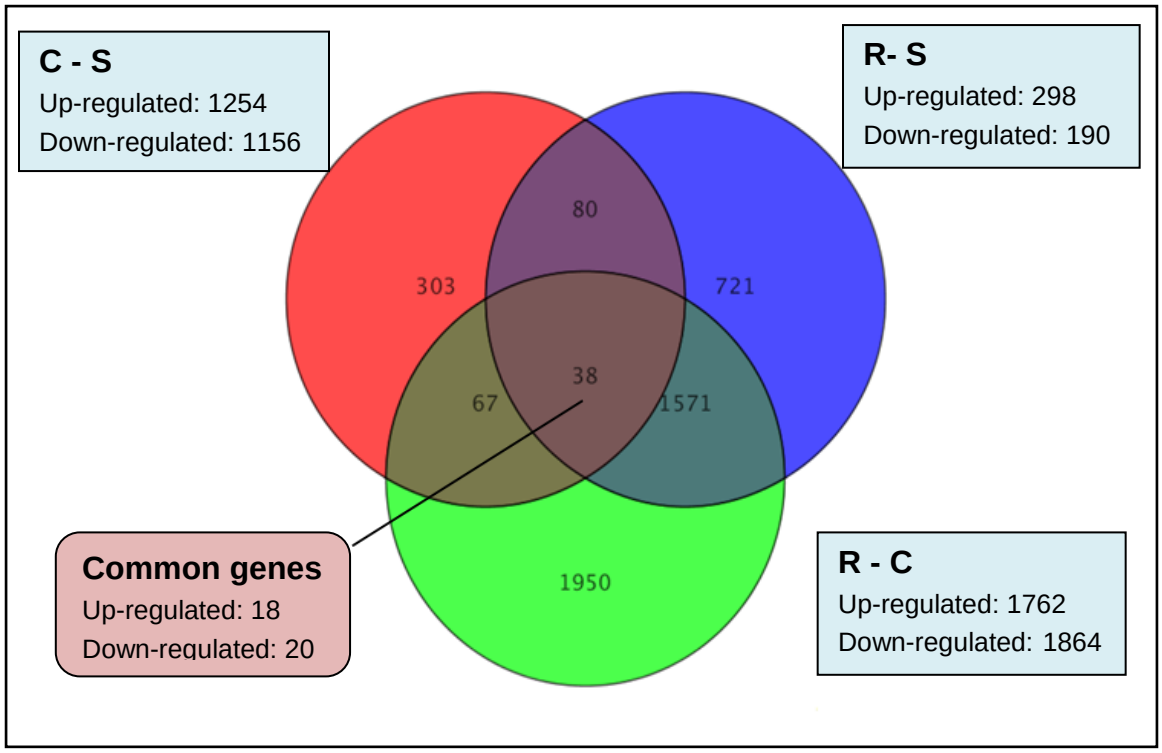

Figure 5.10 Venn diagram of differentially transcribed genes in *Ae. albopictus* for Kuala Lumpur C-S, R-S and R-C (p= 0.05)

Due to major importance of pyrethroid in ongoing vector control interventions against dengue vectors in Malaysia, further investigation was performed on the underlying metabolic resistance mechanisms against permethrin. The KL sample was chosen for this study as it was the only location resistant to permethrin. In addition, the relatively lower level of permethrin resistance in this KL also made it more relevant to compare resistant mosquitoes to non exposed control sample from KL. The big phenotypic difference between the permethrin resistant mosquitoes (100% resistant) and the control non-exposed (13% resistant only) present a higher contrast likely to facilitate the detection of permethrin resistance genes by microarray hybridisation between resistant (R) and Control (C) (R-C). Furthermore, the permethrin resistant mosquitoes (R) were also compared to the

lab susceptible (S) strain (R-S). Data were analyzed according to C-S, R-S and R-C as shown in Figure 5.10.

Genes up-regulated in permethrin resistant samples (R-C)

For this comparison, detoxification genes that were differentially expressed at p=0.01 were analysed. Out of the 433 differentially expressed probes, 269 probes were up-regulated and 164 probes were down-regulated. Several probes belonging to detoxification or likely linked to resistance genes were detected from the list of up-regulated probes including cytochrome P450s, GST, ABC transporters, heat shock protein, oxidaxes, cuticular protein, dehydrogenases and proteases. The most up-regulated gene was a cuticular protein with FC value of 33.79 (Table 5.11) and the likely involvement of a reduced penetration mechanism was further supported by the presence of several other cuticle protein probes (Table 5.11). This is shown by the fact that out of the five top up-regulated genes, 4 belong to cuticular genes. However, probes from the cytochrome P450s were by far the most predominant among the detoxification genes with 20 out of the 27 over-expressed probes. *Aedes albopictus* transcript Aalb_oocyte_GH79BIP02GBWB9, with the closest hit corresponding to CYP6P12 in *Ae. aegypti (89%)* but also 74% similar to CYP6P4 in *An. gambiae,* was the most ever-expressed cytochrome P450 with FC values of 31.79. Interestingly, three probes of another *Ae. albopictus* transcript HQ621849.1 (Genbank) that also has the closest hit to CYP6P12 in *Ae. aegypti* was consistently over-expressed with FC values of 11.36, 11.16 and 8.76. Several other cytochrome P450 transcripts all had the closest hit corresponding to the CYP6P4 gene in *An. gambiae*. One example is the transcript JF317339.1 which has the closest hit to *An. gambiae*. All six probes corresponding to this gene had FC values between 11.18 to 4.94 (Table 5.11). However, transcripts with best hits to both CYP6P12 and CYP6P4 may in fact belong to the ortholog of CYP6P12 and CYP6P4 in *Ae. albopictus*.

Another up-regulated cytochrome P450 was the Aalb_oocyte_GIK0NFC01EFN86 transcript with the closest hit to CYP6ZB1 in *Ae. aegypti*. CYP6ZB1 gene is also represented by *Ae. albopictus* transcript Aalb_oocyte_rep_c13705. All four probes representing this gene have FC values ranging from 9.34 to 5.53 (Table 5.11). Two transcripts: Aalb_oocyte_rep_c3445 and Aalb_oocyte_rep_c28874 corresponding to CYP6AG6 in *Ae. aegypti* were consistently over-expressed with FC value for three probes of 3.46, 3.07 and 3.01. Three probes of *Ae .albopictus* transcript of FJ423553.1 for CYP6A1 gene had similar expression with FC values of 3.074, 3.072 and 3.058. Other cytochrome P450s that were up-regulated were genes with the closest hit to *Ae. aegypti* CYP6Z8 and CYP9J6 (Table 5.11).

The GST that could be observed was the transcript of Aalb_oocyte_rep_c4004 with the closest hit to *Ae. aegypti* GSTT4 (FC 4.34). ABC transporters had FC values ranging from 2.74 to 2.25.

Commonly up-regulated genes between comparisons

Analysis of genes commonly up-regulated in the three comparisons only detects few of the genes observed in the R-C comparison. Only one P450, CYP6AG6 was common to the three comparisons (Table 5.12). Interestingly the antimicrobial peptide Holotricin which was the top up-regulated gene in all the C-S comparison in the three locations was consistently up-regulated in the three comparisons in relation to permethrin resistance. The expression levels consistent for the two probes were associated with possible resistance levels as the highest FC was with R-S (FC 8.9) follow by R-C (FC 4.7) and C-S (FC 2.9) (Table 5.12).

Analysis of the list of probes commonly up-regulated between R-C and R-S but not in C-S (Table 5.13) detected the same genes as reported for R-C further supporting the role of these genes in permethrin resistance. The P450 transcripts corresponding to CYP6P12 were also the top up-regulated in the R-S as in R-C

followed by the transcripts corresponding to CYP6P4. Similarly the cuticular proteins up-regulated in the R-C comparison are also up-regulated in R-S but for all these genes, the fold changes are significantly higher in the R-C comparison than R-S (e.g FC 33.7 in RC and 13.9 for R-S for the top regulated Aalb_oocyte_rep_c16319).

For the Kuala Lumpur R-C and C-S comparison with susceptible VCRU strain, no commonly differentially expressed probes were obtained for FC value of 2 and p = 0.01.

Genes commonly down-regulated between comparisons

Out of the top genes commonly down-regulated between the three comparisons, serine threonine-protein kinase is the highest down-regulated. 4-notrophenylphosphatase seems to be consistently down-regulated with the probes representing this gene to be among the top 4 probes. No detoxification genes could be observed in the commonly down-regulated genes (Table 5.14).

Table 5.11 Probes from detoxification genes & genes linked with resistance up-regulated in *Ae. albopictus* in KL permethrin resistant samples in comparison with KL control (non-exposed) strain. FC = fold change (p = 0.01).

Probe name	Gene-ID	Absolute - FC	Description
CUST_21736_PI427639955	Aalb_oocyte_rep_c16319	33.78784	larval cuticle protein lcp-30
CUST_9941_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	31.79265	cytochrome p450
CUST_21737_PI427639955	Aalb_oocyte_rep_c16319	31.6813	larval cuticle protein lcp-30
CUST_2672_PI427639955	Aalb_oocyte_rep_c18373	27.18452	pupal cuticle
CUST_4200_PI427639955	Aalb_oocyte_rep_c33277	26.79706	pupal cuticle protein
CUST_9942_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	19.85337	cytochrome p450
CUST_93_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	11.36121	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_135_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	11.18272	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_92_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	11.16038	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_134_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	10.30518	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_8072_PI427639955	Aalb_oocyte_GIK0NFC01EFN86 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 *An. gambiae*)	9.342057	cytochrome p450
CUST_8073_PI427639955	Aalb_oocyte_GIK0NFC01EFN86 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 *An. gambiae*)	9.000547	cytochrome p450
CUST_91_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	8.763484	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_20108_PI427639955	Aalb_oocyte_rep_c34982	7.988668	pupal cuticle protein
CUST_858_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 in *An. gambiae*)	5.617168	cytochrome p450
CUST_857_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 in *An. gambiae*)	5.533164	cytochrome p450
CUST_133_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	5.214102	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_123_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	5.03263	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete

			cds
CUST_121_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	4.942938	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds
CUST_122_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	4.935911	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds
CUST_20967_PI427639947	AAEL009131-RA (CYP6Z8 in *Ae. aegypti*)	4.917848	cytochrome P450
CUST_5275_PI427639947	AAEL002638-RA (CYP9J6 in *Ae. aegypti*)	4.819919	cytochrome P450
CUST_32384_PI427639947	AAEL015090-RA	4.640719	heat shock protein
CUST_22330_PI427639955	Aalb_oocyte_rep_c12259	4.375616	serine protease
CUST_20871_PI427639955	Aalb_oocyte_rep_c4004 (as GSTT4 in *Ae. aegypti*)	4.337836	glutathione-s-transferase gst
CUST_2213_PI427639955	Aalb_oocyte_c29950	3.72688	aldehyde oxidase
CUST_11048_PI427639955	Aalb_oocyte_rep_c3445 (CYP6AG6 in *Ae. aegypti*)	3.455315	cytochrome p450
CUST_20740_PI427639947	AAEL008227-RA	3.282475	short-chain dehydrogenase
CUST_20739_PI427639947	AAEL008227-RA	3.270207	short-chain dehydrogenase
CUST_18636_PI427639955	Aalb_oocyte_rep_c28874 (as CYP6AG6 in *Ae. aegypti*)	3.078576	cytochrome p450
CUST_270_PI427639958	FJ423553.1 (CYP6A1)	3.074319	Aedes albopictus cytochrome P450 6A1 mRNA, partial cds
CUST_268_PI427639958	FJ423553.1 (CYP6A1)	3.072208	Aedes albopictus cytochrome P450 6A1 mRNA, partial cds
CUST_269_PI427639958	FJ423553.1 (CYP6A1)	3.057818	Aedes albopictus cytochrome P450 6A1 mRNA, partial cds
CUST_11047_PI427639955	Aalb_oocyte_rep_c3445 (CYP6AG6 in *Ae. aegypti*)	3.015875	cytochrome p450
CUST_29165_PI427639947	AAEL012702-RA	2.740114	ATP-binding cassette sub-family A member 3, putative
CUST_2908_PI427639947	AAEL001101-RA	2.252461	ATP-dependent transporter

Table 5.12 Probes commonly up-regulated in *Ae. albopictus* in Kuala Lumpur R-C, R-S and C-S in comparison with susceptible VCRU strain. FC = fold change. ((p = 0.01).

Probe name	Gene-ID	Absolute – FC			Description
		R-C	R-S	C-S	
Detoxification					
CUST_18637_PI427639955	Aalb_oocyte_rep_c28874 (as CYP6AG6 in *Ae. aegypti*)	2.87335	3.998834	2.847051	cytochrome p450
CUST_18636_PI427639955	Aalb_oocyte_rep_c28874 (as CYP6AG6 in *Ae. aegypti*)	3.078576	3.180108	2.262525	cytochrome p450
Redox/mitochondrial					
CUST_3666_PI427639947	AAEL002504-R	2.366497	2.755429	2.060701	ATP synthase delta chain, mitochondrial
Protein synthesis/metabolism					
CUST_10638_PI427639955	Aalb_oocyte_rep_c40115	3.056011	3.746682	2.248264	protein serine threonine
CUST_10637_PI427639955	Aalb_oocyte_rep_c40115	2.989791	3.678203	2.208736	protein serine threonine
CUST_4331_PI427639955	Aalb_oocyte_rep_c4671	2.398327	4.017792	2.063219	trehalose-6-phosphate synthase 1
CUST_4332_PI427639955	Aalb_oocyte_rep_c4671	2.378469	4.258847	2.034819	trehalose-6-phosphate synthase 1
Transport/ion transport					
CUST_5895_PI427639955	Aalb_oocyte_rep_c7850	2.360499	3.504975	2.548524	sugar transporter
CUST_5894_PI427639955	Aalb_oocyte_rep_c7850	2.15134	2.989625	2.191878	sugar transporter
Immune defence					
CUST_30823_PI427639947	AAEL017536-RA	4.743259	8.955093	2.931466	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide.
CUST_30822_PI427639947	AAEL017536-RA	4.698691	8.92047	2.755124	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide.
Other					
CUST_21247_PI427639947	AAEL007160-RA	4.00375	10.05823	9.207074	ubiquilin 1,2
CUST_15652_PI427639947	AAEL010724-RA	4.382863	5.196907	2.506049	conserved hypothetical protein
CUST_19693_PI427639955	Aalb_oocyte_rep_c28995	2.914513	3.215786	2.049088	protein yellow

Table 5.13 Probes commonly up-regulated in *Ae. albopictus* in Kuala Lumpur R-C and R-S but not C-S in comparison with susceptible VCRU strain. FC = fold change (p = 0.01).

Probe name	Gene-ID	Absolute – FC		Description
		R-C	R-S	
Detoxification				
CUST_9941_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	31.79265	23.29699	cytochrome p450
CUST_9942_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	19.85337	17.06382	cytochrome p450
CUST_92_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	11.16038	10.91106	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_8072_PI427639955	Aalb_oocyte_GIK0NFC01EFN86 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 *An. gambiae*)	9.342057	10.18559	cytochrome p450
CUST_8073_PI427639955	Aalb_oocyte_GIK0NFC01EFN86 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 *An. gambiae*)	9.000547	9.586701	cytochrome p450
CUST_91_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	8.763484	9.445314	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_93_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	11.36121	9.019459	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_135_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	11.18272	8.19095	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_134_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	10.30518	8.136212	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_858_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 in *An. gambiae*)	5.617168	5.90992	cytochrome p450
CUST_857_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 in *An. gambiae*)	5.533164	5.708919	cytochrome p450
CUST_133_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	5.214102	4.164977	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_121_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	4.942938	3.818231	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds

CUST_122_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	4.935911	3.790901	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds
CUST_123_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	5.03263	3.76866	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds
CUST_2213_PI427639955	Aalb_oocyte_c29950	3.72688	3.25696	aldehyde oxidase
CUST_2214_PI427639955	Aalb_oocyte_c29950	3.267967	2.764294	aldehyde oxidase
CUST_20739_PI427639947	AAEL008227-RA	3.270207	2.450861	short-chain dehydrogenase
CUST_20740_PI427639947	AAEL008227-RA	3.282475	2.422125	short-chain dehydrogenase
Cuticular protein				
CUST_2671_PI427639955	Aalb_oocyte_rep_c18373	26.455	14.86722	pupal cuticle
CUST_2672_PI427639955	Aalb_oocyte_rep_c18373	27.18452	14.65215	pupal cuticle
CUST_21737_PI427639955	Aalb_oocyte_rep_c16319	31.6813	14.33227	larval cuticle protein lcp-30
CUST_21736_PI427639955	Aalb_oocyte_rep_c16319	33.78784	13.90945	larval cuticle protein lcp-30
CUST_4200_PI427639955	Aalb_oocyte_rep_c33277	26.79706	10.41951	pupal cuticle protein
Redox/mitochondrial				
CUST_29573_PI427639947	AAEL013637-RA	14.16541	7.384553	homogentisate 1,2-dioxygenase
CUST_29572_PI427639947	AAEL013637-RA	10.99744	7.382413	homogentisate 1,2-dioxygenase
CUST_26955_PI427639947	AAEL014600-RA	14.15808	6.206823	4-hydroxyphenylpyruvate dioxygenase
CUST_23500_PI427639947	AAEL010442-RA	14.85699	6.189439	4-hydroxyphenylpyruvate dioxygenase
CUST_23499_PI427639947	AAEL010442-RA	14.86371	5.969573	4-hydroxyphenylpyruvate dioxygenase
CUST_26954_PI427639947	AAEL014600-RA	15.97921	5.937681	4-hydroxyphenylpyruvate dioxygenase
CUST_23058_PI427639947	AAEL010330-RA	10.24999	5.011088	succinate dehydrogenase
CUST_23057_PI427639947	AAEL010330-RA	8.951039	4.773092	succinate dehydrogenase
CUST_22112_PI427639955	Aalb_oocyte_rep_c39166	7.655777	4.264396	mitochondrial nadh:ubiquinone oxidoreductase esss
CUST_22113_PI427639955	Aalb_oocyte_rep_c39166	6.337713	4.17311	mitochondrial nadh:ubiquinone oxidoreductase
CUST_7016_PI427639955	Aalb_oocyte_GH79BIP01ALUD2	4.781242	3.520882	nad dehydrogenase
CUST_10975_PI427639955	Aalb_oocyte_rep_c693	4.691548	3.504764	succinate dehydrogenase
CUST_329_PI427639955	Aalb_oocyte_rep_c10161	5.185031	3.357888	oxidoreductase
CUST_330_PI427639955	Aalb_oocyte_rep_c10161	5.077024	3.343754	oxidoreductase
CUST_10976_PI427639955	Aalb_oocyte_rep_c693	4.498606	3.290983	succinate dehydrogenase
Lipid/carbohydrate synthesis/metabolism				
CUST_16264_PI427639947	AAEL008160-RA	2.596339	3.294166	fatty acid synthase

CUST_33999_PI427639947	AAEL014863-RE	8.612796	2.999631	glycogenin
Transport/ion transport				
CUST_27761_PI427639947	AAEL012480-RA	7.897954	3.964984	sodium/calcium exchanger
CUST_11159_PI427639947	AAEL005496-RA	3.24924	2.960566	zinc/iron transporter
CUST_2908_PI427639947	AAEL001101-RA	2.252461	2.794819	ATP-dependent transporter
Immune defence				
CUST_30823_PI427639947	AAEL017536-RA	4.743259	8.955093	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide.
CUST_30822_PI427639947	AAEL017536-RA	4.698691	8.92047	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide.
Cell organization and biogenesis				
CUST_12860_PI427639947	AAEL006872-RA	5.255193	2.721025	calponin/transgelin
CUST_12859_PI427639947	AAEL006872-RA	5.205531	2.642643	calponin/transgelin
CUST_22868_PI427639947	AAEL008303-RA	8.171104	2.636543	calponin/transgelin
CUST_16701_PI427639955	Aalb_oocyte_rep_c8489	6.059429	2.164696	calponin/transgelin
Signalling				
CUST_3808_PI427639947	AAEL001963-RA	8.052182	2.351239	protein serine/threonine kinase, putative
CUST_3807_PI427639947	AAEL001963-RA	6.82989	2.301584	protein serine/threonine kinase, putative
Other				
CUST_22221_PI427639955	Aalb_oocyte_GH79BIP02GFPJD	93.00824	63.5122	hypothetical protein AaeL_AAEL006255 [Aedes aegypti]
CUST_9840_PI427639955	Aalb_oocyte_rep_c38702	80.88271	59.32635	pre-mrna-splicing factor cwc15
CUST_9839_PI427639955	Aalb_oocyte_rep_c38702	70.23261	59.00159	pre-mrna-splicing factor cwc15
CUST_32777_PI427639947	AAEL014279-RA	60.71153	47.58711	glycosyltransferase
CUST_11466_PI427639947	AAEL003235-RB	28.04045	17.89262	conserved hypothetical protein
CUST_35438_PI427639947	AAEL013083-RA	15.83911	12.42848	conserved hypothetical protein
CUST_16754_PI427639955	Aalb_oocyte_rep_c6603	8.97192	10.0589	translation initiation factor if-2
CUST_16755_PI427639955	Aalb_oocyte_rep_c6603	8.967837	9.370801	translation initiation factor if-2
CUST_8673_PI427639947	AAEL003041-RA	12.3796	5.362907	conserved hypothetical protein
CUST_19861_PI427639955	Aalb_oocyte_rep_c14000	9.571454	5.124756	lethal essential for life l2efl
CUST_1926_PI427639955	Aalb_oocyte_rep_c13435	13.92644	4.290054	centromeric protein
CUST_22178_PI427639955	Aalb_oocyte_GH79BIP01B00UY	2.442447	4.281398	alpha-glucosidase
CUST_1925_PI427639955	Aalb_oocyte_rep_c13435	11.03708	3.789974	centromeric protein
CUST_1933_PI427639955	Aalb_oocyte_rep_c22	3.783072	3.523356	40s ribosomal protein s12
CUST_7474_PI427639955	Aalb_oocyte_rep_c355	4.02747	3.309502	mitochondrial f0 atp synthase d

CUST_20098_PI427639955	Aalb_oocyte_GH79BIP02FHQER	4.230109	3.136519	hypothetical protein AaeL_AAEL003107 [Aedes aegypti]
CUST_6742_PI427639947	AAEL003931-RA	5.312818	3.109783	conserved hypothetical protein
CUST_8220_PI427639947	AAEL005179-RA	4.661499	2.766373	hypothetical protein
CUST_17134_PI427639947	AAEL008771-RA	3.764787	2.764974	conserved hypothetical protein
CUST_6741_PI427639947	AAEL003931-RA	5.441568	2.726682	conserved hypothetical protein
CUST_19345_PI427639955	Aalb_oocyte_rep_c2797	3.364845	2.687364	membrane-associated lps-inducible tnf alpha factor protein
CUST_34723_PI427639947	AAEL014717-RA	2.427558	2.600744	adiponectin receptor
CUST_19182_PI427639955	Aalb_oocyte_rep_c51687	3.140034	2.584052	isoform a
CUST_18045_PI427639955	Aalb_oocyte_rep_c16111	6.615882	2.57985	protein takeout
CUST_18044_PI427639955	Aalb_oocyte_rep_c16111	5.842744	2.540345	protein takeout
CUST_10354_PI427639955	Aalb_oocyte_rep_c25391	4.260937	2.371017	odorant-binding protein
CUST_10879_PI427639955	Aalb_oocyte_rep_c8039	3.347203	2.333757	neurochondrin homolog
CUST_4433_PI427639947	AAEL002897-RA	3.955691	2.304023	conserved hypothetical protein
CUST_5530_PI427639955	Aalb_oocyte_rep_c11888	2.102471	2.222477	cation proton antiporter
CUST_10353_PI427639955	Aalb_oocyte_rep_c25391	3.686304	2.166117	odorant-binding protein
CUST_14782_PI427639955	Aalb_oocyte_c31200	6.098867	2.113651	isoform g
CUST_7545_PI427639955	Aalb_oocyte_rep_c39781	2.957181	2.099255	mitochondrial processing peptidase beta subunit
CUST_14364_PI427639955	Aalb_oocyte_rep_c42411	2.151837	2.037278	upf0327 protein c1orf151-like
CUST_14365_PI427639955	Aalb_oocyte_rep_c42411	2.021297	2.023597	upf0327 protein c1orf151-like
Unknown				
CUST_9943_PI427639947	AAEL005122-RA	22.95028	21.21412	---NA---
CUST_15740_PI427639955	Aalb_oocyte_GH79BIP01B0XPM	16.09726	8.181257	---NA---
CUST_15741_PI427639955	Aalb_oocyte_GH79BIP01B0XPM	16.78767	7.950053	---NA---

Table 5.14 Top 20 commonly down-regulated probes in *Ae. albopictus* in Kuala Lumpur R-C, R-S and C-S in comparison with susceptible VCRU strain. FC = fold change (p = 0.01).

Probe name	Gene-ID	Absolute – FC			Description
		R-C	R-S	C-S	
CUST_48_PI427639955	Aalb_oocyte_rep_c14495	7.339163	41.6069	6.373849	serine threonine-protein kinase rio2
CUST_664_PI427639955	Aalb_oocyte_rep_c171	12.64692	27.38883	4.633462	4-nitrophenylphosphatase
CUST_20261_PI427639947	AAEL007097-RA	9.757583	25.71153	4.597303	4-nitrophenylphosphatase
CUST_663_PI427639955	Aalb_oocyte_rep_c171	12.22798	27.869	4.527259	4-nitrophenylphosphatase
CUST_18445_PI427639955	Aalb_oocyte_GH79BIP02I1W9U	9.595177	20.37518	4.223555	---NA---
CUST_18444_PI427639955	Aalb_oocyte_GH79BIP02I1W9U	8.633107	16.1546	4.185411	---NA---
CUST_19484_PI427639955	Aalb_oocyte_GH79BIP01AY0KB	7.326617	12.41895	4.178252	lethal essential for life l2efl
CUST_9025_PI427639955	Aalb_oocyte_GH79BIP01A3RBT	4.785714	7.587472	3.842881	---NA---
CUST_22690_PI427639955	Aalb_oocyte_rep_c838	6.078961	13.82323	3.604309	lethal essential for life l2efl
CUST_22691_PI427639955	Aalb_oocyte_rep_c838	5.92025	12.18109	3.562234	lethal essential for life l2efl
CUST_3019_PI427639955	Aalb_oocyte_rep_c21768	5.212205	7.53403	2.63336	cysteine-rich venom
CUST_16355_PI427639955	Aalb_oocyte_rep_c13318	7.036968	8.519937	2.626761	---NA---
CUST_16354_PI427639955	Aalb_oocyte_rep_c13318	5.637769	8.523928	2.481373	---NA---
CUST_24391_PI427639947	AAEL010094-RA	3.47918	6.053329	2.41163	cyclin b
CUST_6286_PI427639955	Aalb_oocyte_rep_c11762	4.672631	2.577583	2.231933	isoform a
CUST_6665_PI427639947	AAEL005495-RA	2.493337	2.810299	2.127734	phospholipid-transporting ATPase 1 (aminophospholipid flippase 1)
CUST_22063_PI427639955	Aalb_oocyte_rep_c13096	3.027491	6.691543	2.104775	hypothetical protein AaeL_AAEL004104 [Aedes aegypti]
CUST_4104_PI427639947	AAEL002565-RA	8.036596	2.915349	2.086632	titin
CUST_14783_PI427639955	Aalb_oocyte_c31200	5.933088	2.157974	2.035758	isoform g
CUST_20890_PI427639955	Aalb_oocyte_rep_c10794	2.639613	4.639919	2.021467	leucine rich protein

5.3.5.3 Comparison of *Aedes albopictus* control strains from PG and JB, KL resistant strain against susceptible VCRU lab strain

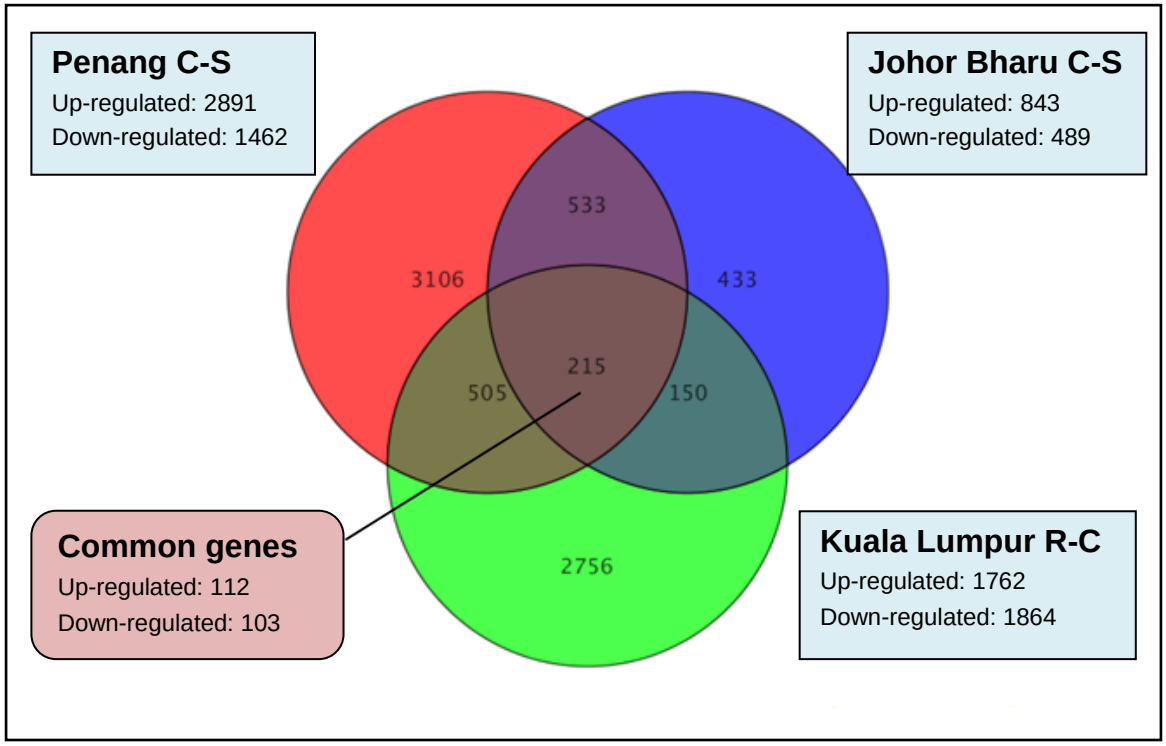

Figure 5.11 Venn diagram of differentially transcribed genes in *Ae. albopictus* for PG C-S, JB C-S and KL R-C (p= 0.05)

This comparison was done to access Permethrin resistance across Malaysia. Because the R-C comparison of KL appeared representative of the list of genes conferring permethrin resistance, attempt was made to detect the genes involved in resistance against other insecticide classes than pyrethroids by comparing the R-C to the C-S from other locations. The common genes show that the non-permethrin resistant genes are only present in PG and JB. From these comparisons, the list of genes commonly up-regulated between KL R-C and the C-S of PG and JB contains the similar main cytochrome P450s observed in R-C KL notably CYP6P12 and CYP6P4 or CYP6ZB1 but always with a significantly higher expression in KL R-C than in the C-S (Table 5.15). For example, the transcript (Aalb_oocyte_GH79BIP02GBWB9) corresponding to CYP6P12 has an FC of 31.79 in KL R-C but only FC of 3.45 in PG and 10.51 in JB. Similar trend

was observed for other P450 genes. However the cuticular proteins highly expressed in R-C KL are not over-expressed in the C-S from JB and PG.

5.3.5.4 GO enrichment analysis

Genes or entities that were considered as significantly differentially expressed were used for Gene Ontology (GO) enrichment analysis using Blast2GO software (BioBam Bioinformatics S.L., Valencia, Spain). An attempt was made but unfortunately due to incomplete data (incomplete genome of *Ae. albopictus*, the GO enrichment analysis could not give reliable results.

Table 5.15 Probes commonly up-regulated in *Ae. albopictus* in PG C-S, JB C-S and KL R-C in comparison with susceptible VCRU strain. FC = fold change (p = 0.05).

Probe name	Gene-ID	Absolute – FC			Description
		PG C-S	JB C-S	KL R-C	
Detoxification					
CUST_1663_PI427639955	Aalb_oocyte_rep_c11155 (GSTT3 in *Ae. aegypti*)	6.491828	10.19155	16.47452	glutathione-s-transferase gst
CUST_9941_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	3.453267	10.51562	31.79265	cytochrome p450
CUST_9942_PI427639955	Aalb_oocyte_GH79BIP02GBWB9 (as CYP6P12 in *Ae.aegypti*)	3.299378	7.390336	19.85337	cytochrome p450
CUST_92_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	3.293268	3.742736	11.16038	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_93_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	3.072198	3.764997	11.36121	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_857_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 in *An. gambiae*)	2.800269	4.632875	5.533164	cytochrome p450
CUST_91_PI427639958	HQ621849.1 (as CYP6P12 in *Ae.aegypti*)	2.794235	3.133532	8.763484	Aedes albopictus isolate P18 cytochrome P450 mRNA, partial cds
CUST_134_PI427639958	JF317339.1 (as CYP6P4 in *An. gambiae*)	2.537406	3.029287	11.18272	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_21999_PI427639955	Aalb_oocyte_rep_c13281 (as CYP6Z8 in *Ae. aegypti*)	2.390325	3.935278	6.690396	cytochrome p450
CUST_858_PI427639955	Aalb_oocyte_rep_c13705 (as CYP6ZB1 in *Ae. aegypti* and CYP6P4 in *An. gambiae*)	2.278028	2.729131	10.30518	Aedes albopictus clone 22v2 cytochrome P450 mRNA, complete cds
CUST_122_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	2.271363	4.870811	5.617168	cytochrome p450
CUST_121_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	2.048554	2.060227	4.935911	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds
CUST_123_PI427639958	JF317338.1 (as CYP6P4 in *An. gambiae*)	2.030469	2.023919	4.942938	Aedes albopictus clone 22v1 cytochrome P450 mRNA, complete cds

Redox/mitochondrial					
CUST_27482_PI427639947	AAEL012614-RA	3.279994	4.252524	2.293867	NADP-specific isocitrate dehydrogenase
CUST_1713_PI427639955	Aalb_oocyte_rep_c25929	3.188376	3.580843	3.379762	atp-citrate synthase
CUST_2367_PI427639955	Aalb_oocyte_rep_c2659	2.882715	5.411058	2.731306	electron transfer flavoprotein-ubiquinone oxidoreductase
CUST_18132_PI427639955	Aalb_oocyte_rep_c16323	2.474228	2.606252	2.071727	d-amino acid oxidase
CUST_18133_PI427639955	Aalb_oocyte_rep_c16323	2.396793	2.444541	2.179393	d-amino acid oxidase
CUST_10975_PI427639955	Aalb_oocyte_rep_c693	2.329191	2.343106	4.691548	succinate dehydrogenase
CUST_7271_PI427639947	AAEL004086-RB	2.318355	2.131969	4.196262	aldo-keto reductase
CUST_10162_PI427639955	Aalb_oocyte_c21124	2.318003	10.24194	3.255944	reverse transcriptase
CUST_10976_PI427639955	Aalb_oocyte_rep_c693	2.293186	2.313377	4.498606	succinate dehydrogenase
Transport/ion transport					
CUST_4570_PI427639947	AAEL001626-RA	5.074514	9.249521	3.525148	zinc/iron transporter
CUST_5895_PI427639955	Aalb_oocyte_rep_c7850	3.644206	2.972166	2.360499	sugar transporter
CUST_3797_PI427639955	Aalb_oocyte_GH79BIP01BRT27	2.477897	2.126668	2.155722	sugar transporter
Immune defence					
CUST_1492_PI427639947	AAEL000627-RA	13.8122	8.770173	27.16076	Cecropin-A Precursor
CUST_1491_PI427639947	AAEL000627-RA	13.26494	7.682735	35.59871	Cecropin-A Precursor
CUST_30823_PI427639947	AAEL017536-RA	12.97018	12.63358	4.743259	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide.
CUST_30822_PI427639947	AAEL017536-RA	12.96873	12.4153	4.698691	Holotricin, Glycine Rich Repreat Protein (GRRP), Anti-Microbial Peptide.
CUST_10110_PI427639947	AAEL004522-RA	10.97845	8.50843	32.19684	Gambicin,Anti-Microbial Peptide.
CUST_10109_PI427639947	AAEL004522-RA	10.32434	8.982585	37.55361	Gambicin,Anti-Microbial Peptide.
Nucleobase, nucleoside, nucleotide ano nucleic acid metabolism					
CUST_21266_PI427639955	Aalb_oocyte_rep_c11036	6.61479	11.35482	6.609391	activating signal cointegrator 1
Signalling					
CUST_7860_PI427639947	AAEL004967-RA	3.433731	2.675341	2.444301	myo inositol monophosphatase
Other					
CUST_21247_PI427639947	AAEL007160-RA	12.22205	12.42675	4.00375	ubiquilin 1,2
CUST_29053_PI427639947	AAEL014534-RA	11.38186	15.70106	7.522248	conserved hypothetical

					protein
CUST_26079_PI427639947	AAEL011897-RA	11.28304	4.267724	23.27615	conserved hypothetical protein
CUST_26078_PI427639947	AAEL011897-RA	9.117275	4.930036	26.12517	conserved hypothetical protein
CUST_31742_PI427639947	AAEL013464-RA	8.631622	16.13228	8.16907	conserved hypothetical protein
CUST_9687_PI427639947	AAEL005490-RA	5.538354	3.771485	2.401847	microsomal dipeptidase
CUST_8972_PI427639947	AAEL004534-RA	5.218485	4.853004	5.176921	conserved hypothetical protein
CUST_20302_PI427639955	Aalb_oocyte_rep_c7983	5.18412	2.566623	5.186282	hypothetical protein AaeL_AAEL008106 [Aedes aegypti]
CUST_1644_PI427639955	Aalb_oocyte_GIK0NFC01D9L0C	4.550805	11.38742	6.151239	grx
CUST_20301_PI427639955	Aalb_oocyte_rep_c7983	4.400115	2.300072	4.60815	hypothetical protein AaeL_AAEL008106 [Aedes aegypti]
CUST_23268_PI427639947	AAEL010730-RA	4.109683	4.683131	2.720561	conserved hypothetical protein
CUST_27446_PI427639947	AAEL011358-RA	4.026766	6.494739	10.01657	origin recognition complex subunit
CUST_6845_PI427639955	Aalb_oocyte_rep_c12444	3.547347	2.324054	2.149078	isoform b
CUST_1090_PI427639955	Aalb_oocyte_rep_c30571	3.140454	2.03813	4.206515	hypothetical protein AaeL_AAEL012849 [Aedes aegypti]
CUST_8342_PI427639955	Aalb_oocyte_rep_c12061	3.133889	4.448216	2.677005	isoform a
CUST_21685_PI427639955	Aalb_oocyte_rep_c6627	3.004621	9.801999	3.365939	pre-mrna-splicing factor slu7
CUST_30453_PI427639947	AAEL011837-RA	2.705691	4.539245	4.598143	cAMP-dependent protein kinase catalytic subunit
CUST_324_PI427639947	AAEL000166-RA	2.49527	2.524443	3.072275	conserved hypothetical protein
CUST_30464_PI427639947	AAEL012957-RA	2.493846	2.937875	2.110767	conserved hypothetical protein
CUST_24288_PI427639947	AAEL008401-RA	2.450439	3.09814	2.295036	conserved hypothetical protein
CUST_17087_PI427639955	Aalb_oocyte_rep_c4801	2.438792	2.576748	2.078534	adp-ribosylation factor-like protein 6-interacting protein 4
CUST_10161_PI427639955	Aalb_oocyte_c21124	2.405897	9.8016	3.079377	reverse transcriptase
CUST_3108_PI427639955	Aalb_oocyte_GH79BIP01A2O1W	2.322243	2.341644	2.245699	hypothetical protein AaeL_AAEL001095 [Aedes

					aegypti]
CUST_17086_PI427639955	Aalb_oocyte_rep_c4801	2.231353	2.572714	2.028761	adp-ribosylation factor-like protein 6-interacting protein 4
CUST_34722_PI427639947	AAEL014717-RA	2.169878	5.014514	2.263151	adiponectin receptor
CUST_19458_PI427639947	AAEL010411-RA	2.069104	3.477532	2.270014	dual specificity protein phosphatase
CUST_19457_PI427639947	AAEL010411-RA	2.054042	3.962242	2.787607	dual specificity protein phosphatase
CUST_11420_PI427639947	AAEL005308-RA	2.030575	3.640688	3.066409	pyruvate dehydrogenase
CUST_34723_PI427639947	AAEL014717-RA	2.025352	5.351576	2.427558	adiponectin receptor
CUST_1933_PI427639955	Aalb_oocyte_rep_c22	2.022034	6.944415	3.783072	40s ribosomal protein s12

5.3.6 Validation of candidate genes through qRT-PCR

Thirteen genes over-expressed through microarray analysis were chosen for validation using qRT-PCR. After several attempts of qRT-PCR, CYP6N9, CYP6Z6 and CYP6P4 were removed from the data analysis because their relative FC expression could not be obtained. This could be due to the primer designed. Among the eleven candidate genes selected for qRT-PCR validation six were cytochrome P450s (CYP6N3, CYP6AE1, CP6P12, CYP6M6, CYP9J17, CYP6M2), two glutathione-S transferases (GSTT3, GSTDI), one ABC transporter (ABC transporter A) and two short-chain dehydrogenises SCD15871 (Aalb_oocyte_rep_c15871) and SCD01845 (AAEL001845-RA) (Figure 5.12). Samples from Kota Bharu (KB) were also tested in the qRT-PCR.

All primer pairs tested had efficiencies between 90% and 110%. A significant over-expression in all four populations was confirmed for 10 genes except for CYP9M6 when their relative expression was compared between the four populations and susceptible VCRU strain after normalization with two housekeeping genes [tubulin (AAEL013229-RA) and ribosomal protein S7 (RSP7) (AAEL009496-RA)] (Figure 5.12).

Different locations showed different fold changes for all 11 genes. Overall, cytochrome P450 CYP6N3 was the most consistently top over-expressed gene across the four locations with FC value of 201.40 in PG, 39.82 in KL, 45.31 in JB and 55.06 in KB. Short-chain dehydrogenase SCD15871was also amongst the top over-expressed in PG (FC 29.26), JB (FC 312.39) and KB (FC 69.71) except for in KL which was under-expressed with FC value of 0.85. In Penang, the highest FC is observed for CYP6N3 followed by CYP6M2 with 44.65 fold up-regulation and CP6AE1 with 27.93 fold up-regulation. In Kuala Lumpur, CYP6M2 was also up-regulated with FC of 31.69. CYP6AE1 was also up-regulated in JB and KB with 10.90 and 6.80 fold up-regulation respectively. No over-transcription was observed for CYP9M6 in KL and JB with FC values of 0.75 and 0.46 respectively.

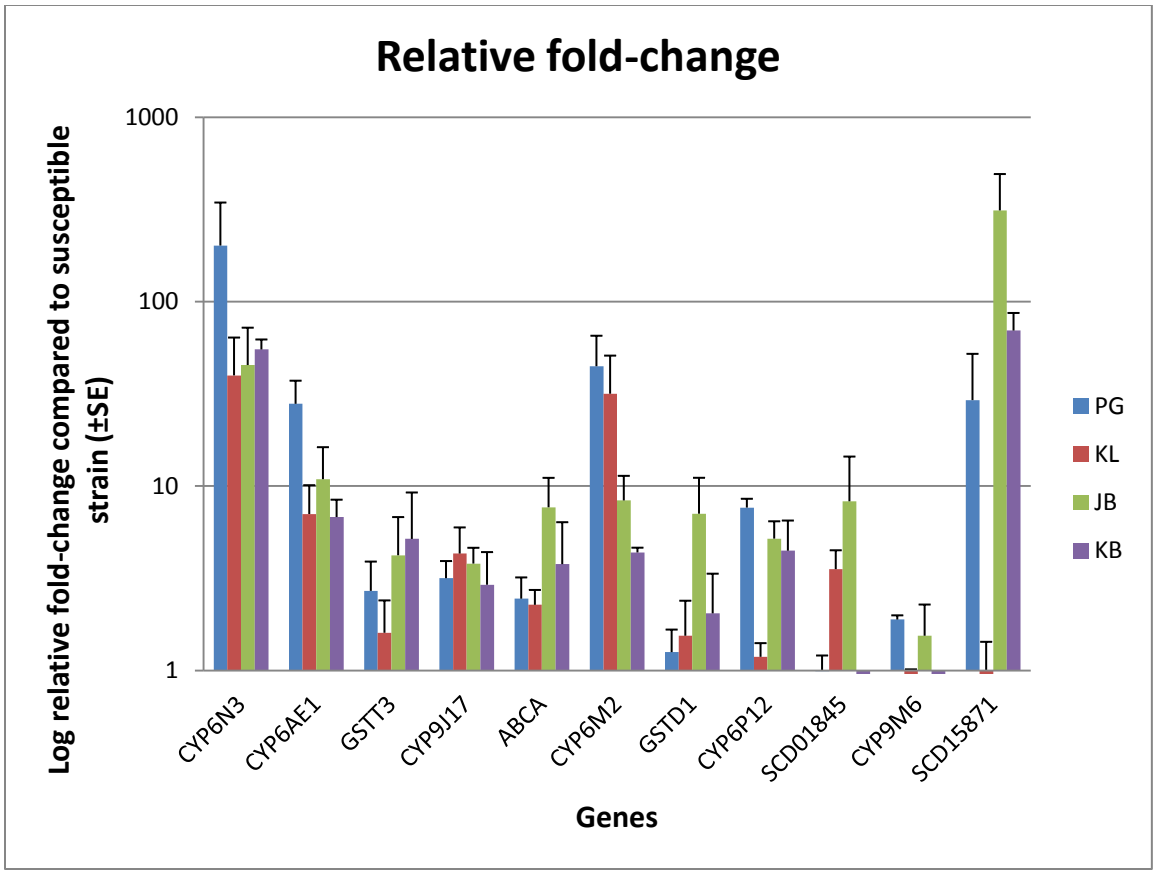

Figure 5.12 Relative fold-change of candidate genes in *Ae. albopictus* from qRT-PCR analysis.

5.4 Discussion

This study for the first time in Malaysia characterised the underlying mechanisms conferring resistance to insecticides in the dengue vector *Ae. albopictus*. Through the sequencing of target-site resistance genes and a genome-wide transcription analysis using microarray, this study has provided a broader insight into the molecular mechanisms generating the observed resistance phenotypes in this important vector.

DDT and Pyrethroid resistance are not associated with knockdown resistance mechanisms

The sequencing of portions of the VGSC gene likely to harbour *kdr* mutations revealed that there is no *kdr* mutation in the four *Ae. albopictus* populations across Malaysia. This shows that knockdown resistance plays no role in the observed DDT resistance and even the pyrethroid resistance observed in KL. This absence is not too surprising when taking into consideration the cross resistance pattern between DDT and pyrethroids in this species across Malaysia. Indeed, although DDT resistance was consistently detected in the four locations, resistance to pyrethroids was only detected in KL where a moderate resistance to permethrin was recorded. The lack of cross-resistance pyrethroids/DDT supports the absence of knockdown resistance mechanism in these populations as its presence would have most likely confer a cross resistance between DDT and pyrethroids since these two insecticide classes have the voltage-gated sodium channel gene as the same target. Such cross resistance conferred by *kdr* mutations is common in other mosquito species such as *Ae. aegypti* (Harris et al., 2010, Vontas et al., 2012) or *An. gambiae* (Ranson et al., 2000, Martinez-Torres et al., 1998).

This absence of *kdr* mutation in *Ae. albopictus* in Malaysia is also in line with previous studies which could not detect such mutations in other populations of this species worldwide (Tantely et al., 2010, Liu et al., 2006, Vontas et al., 2012). In this respect, *Ae. albopictus* is similar to other insect species such as the

malaria mosquito *An. funestus* for which the knockdown resistance plays no or only limited role in observed DDT and pyrethroid resistance (Cuamba et al., 2010, Djouaka et al., 2011). However, the first report of the detection of a *kdr* mutation in Singapore in 2011 (Kasai *et al.*, 2011) shows that such mutation should continually be monitored in *Ae. albopictus* populations in Malaysia. Indeed Kasai *et al.* (2011) reported that 53.8% of the Singapore *Ae. albopictus* population were homozygous for F1534C *kdr* mutation and the estimated allele frequency for the mutation was 73.1%. With such high frequency, it will not be surprising that through gene flow, the F1534C mutation migrates to Malaysia in near future. Nevertheless, the absence of this F1534C mutation in Malaysia even in Johor Bharu which is closest to Singapore, suggests that possible barriers to gene flow may exist. This is more evident because Malaysia is only separated from Singapore by the Johor-Singapore Causeway (1km long).Furthermore, analysis of the genetic variability of the VGSC fragments suggests that no other *kdr* mutations is present in *Ae. albopictus* even beyond the fragments sequenced in this study which mainly covered the mutations commonly found in *Ae. aegypti*. This suggestion is supported by the high genetic diversity observed for the two VGSC fragments and a complete lack of correlation between haplotypes and resistance phenotypes. This absence of a signature of selection on the VGSC gene therefore suggests that there is no *kdr* mutation in these *Ae. albopictus* populations from Malaysia or that if there is any in other regions of this gene, it will be only be playing a minor role. This is similar to patterns of genetic variability also observed in the malaria vector *An. funestus* where the *kdr* mutation is absent despite DDT and pyrethroid resistance (Djouaka et al., 2011, Morgan et al., 2010).

The absence of *kdr* mutation in *Ae. albopictus* is in strong contrast to the four different such mutations present in *Ae. aegypti* worldwide and two found in Malaysia (V1016G and F1534C) (Sections 4.3.2.1 and 4.3.5). This difference is in line with the contrast observed in the resistance profiles of the two species in Malaysia with high DDT and pyrethroid resistance observed in *Ae. aegypti* populations but only DDT resistance mainly observed in *Ae. albopictus*.

Therefore, it is likely that *Ae. albopictus* is not under the same selection pressure as *Ae. aegypti* probably because of difference in their ecological niche. The same situation between the two species is observed worldwide (Vontas *et al.*, 2012).

Investigation of other target site resistance mechanism focused on the sequencing of the Acetylcholinesterase (*Ace-1*) gene in which mutations may lead to resistance to carbamates/organophosphates in other insects such as in *An. gambiae* (Weill *et al.*, 2004). However, the experiment failed due to indels in the sequences that were obtained. To overcome this problem, the next step should be cloning of the sequences to obtain good results. Another target site resistance mechanism that could be explored is the possible presence of RDL mutation as dieldrin resistance is present in the Malaysian *Ae. albopictus* populations and as observed in other populations of this species such as in La Reunion (Tantely *et al.*, 2010) this resistance is likely caused by the presence of the A302S RDL mutation.

Metabolic resistance mechanism is driving resistance in *Ae. albopictus* in Malaysia

The genome-wide transcriptional analyses carried out using microarray provided plenty evidences that the metabolic resistance was the main mechanism conferring resistance to insecticides in *Ae. albopictus* across Malaysia. This was supported by the over-expression of many genes belonging to detoxification gene families across the three locations tested when comparing them to the laboratory susceptible VCRU strain. The most preeminent detoxification gene family was the cytochrome P450 genes which were the only detoxification family commonly over-expressed in the three locations. Among these cytochrome P450s, several transcripts of *the Ae. albopictus* gene CYP6N3 or matching its ortholog in *Ae. aegypti* CYP6N9, were the most over-expressed from the list of commonly expressed genes in the three locations or only between two locations. CYP6N3 is the ortholog of CYP6N1 which has been associated with pyrethroid

resistance in an *An. funestus* from Malawi but only with low fold change of around 2 (Riveron *et al.*, 2013). The higher over-expression of this gene in PG which is fully susceptible to pyrethroids suggests that CYP6N3 could be rather conferring resistance to other insecticides particularly bendiocarb which is the main insecticide for which the PG population is resistant to. Indeed, the PG population is fully susceptible to both type I and II pyrethroids and also to the organophosphate malathion, only very moderately resistant to DDT (96.8% mortality for females) but exhibit a high resistance level to bendiocarb with only 28% mortality. It will be interesting in future to further assess the role of CYP6N3 with functional analyses such as transgenic expression in *Drosophila melanogaster* and metabolic assays with recombinant CYP6N3 enzyme as done in other mosquito species (Riveron et al., 2013, Daborn et al., 2007) (Stevenson *et al.*, 2012).

Overall, several P450 genes belonging to the CYP6 family were over-expressed in the C-S across Malaysia including CYP6N3, CYP6P12, CYP6Z6 CYP6AG6 while only few cytochrome P450s from the CYP9 family were over-expressed. This is in strong contrast to the other dengue vector *Ae. aegypti* for which metabolic resistance from cytochrome P450s is conferred mainly by genes belonging to the CYP9 family such as observed in Chapter 4 across Malaysia or in previous studies worldwide (Strode et al., 2008, Marcombe et al., 2009, Bariami et al., 2012, Vontas et al., 2012). The pre-eminence of CYP6 family in insecticide resistance in *Ae. albopictus* is rather similar to patterns observed for *Anopheles* mosquitoes such as *An. gambiae* or *An. funestus* (David et al., 2005, Muller et al., 2008a, Wondji et al., 2009) (Riveron *et al.*, 2013). This difference between *Ae. albopictus* and *Ae. aegypti* suggests that the speciation between these two species is extensive and have led to significant differences in their metabolic responses to xenobiotic-related stress.

A surprising observation in this study was the highest over-expression obtained for the three C-S comparisons for probes from the antimicrobial peptide halotricin a glycine rich repeat protein (GRRP). The potential association of this gene with

insecticide resistance was further supported as it is also significantly over-expressed in the comparison between the permethrin resistant and the control population from KL (R-C) but also the permethrin resistant and the susceptible VCRU strain (R-S). Over-expression of immune response genes in insecticide resistant mosquito strains has previously been reported notably in *An. gambiae* when the defensin and cecropin genes were found up-regulated in permethrin (RSP strain) and DDT (ZANU) resistant strains (Vontas *et al.*, 2005).

Pyrethroid resistance is likely under the control of cytochrome P450s and a reduced cuticle penetration mechanism

To further identify those genes closely associated with this resistance. The microarray analysis strongly supported that permethrin resistance in *Ae. albopictus* in KL was conferred by two mechanisms, firstly a reduced penetration through cuticle and secondly a detoxification through cytochrome P450 genes.

A more thorough analysis of the transcription pattern associated with permethrin resistance was carried out. The genes associated with permethrin resistance were further detected by comparing the permethrin resistant strain in Kuala Lumpur to non-exposed Kuala Lumpur samples. The list of significantly up-regulated detoxification or resistance associated genes detected through this comparison suggests that when the resistance to a specific insecticide is not high in a population and a significant difference still exists in the resistance phenotype of the fully resistant (after exposure to insecticide) and the non exposed mosquitoes (Control) as it was the case in the permethrin resistance in KL, the R-C comparison could provide the best approach to detect the genes involved in such resistance. Previous attempts using such R-C comparison have not been too successful notably in malaria vectors such as *An. funestus* (Riveron *et al.*, 2013) or *An. gambiae* (Tene *et al.*, 2013) mainly because resistance was already high in the population and the phenotypic difference between the resistant mosquitoes after exposure (R) was minimal than that of control non-exposed

population. The presence of a reduced cuticle penetration mechanism for permethrin resistance was supported by the fact that several probes belonging to cuticular protein genes were consistently over-expressed in the KL R-C comparison. The possible role of reduced penetration was further supported because of consistency of the expression of these probes but also their higher FC with the highest over-expressed gene been always a cuticle protein either at $P<0.05$ (the transcript Aalb_oocyte_GH79BIP02H77ZJ with FC of 77.8) or at $P<0.01$ (Aalb_oocyte_rep_c16319 with FC of 33.7) observed. Over-expression of cuticular protein genes has previously been reported in several studies on insecticide resistance mechanisms such as in *An. gambiae* (Vontas et al., 2005, Djouaka et al., 2008) or in *An. funestus* (Riveron *et al.*, 2013). However the level of fold change observed in this *Ae. albopictus* populations far exceeds that observed in these other mosquito species suggesting the reduced penetration mechanism could be playing a more important role in this *Ae. albopictus* population. Reduced penetration through cuticular thickening has not been previously reported as the cause of insecticide resistance in *Ae. albopictus* but in *An. funestus,* the cuticle thickening was associated with pyrethroid resistance (Wood *et al.*, 2010). This mechanism was less present in *Ae. aegypti* populations in Malaysia as no significant cuticular protein was highly expressed from the microarray experiments suggesting that reduced cuticular penetration is less involved in the resistance observed in this species in Malaysia.

Even though cuticular protein was highly over-expressed in the permethrin resistant comparison, cytochrome P450 genes were by far the most abundantly over-expressed gene family suggesting that these genes confer permethrin resistance in *Ae. albopictus* in KL. Probes from several transcripts showing the best BLAST hits to either CYP6P12 in *Ae. aegypti* or to CYP6P4 in *An. gambiae* were consistently the most over-expressed of the P450s. Because CYP6P12 and CYP6P4 are actually othologous genes, it most likely that all these transcripts in *Ae. albopictus* belong to a same gene which is also ortholog of CYP6P12 and CYP6P4. It is not excluded that these transcripts could also belong to duplicated version of these genes as observed in *An. funestus* where the CYP6P4 is

duplicated in two copies CYP6P4a and CYP6P4b (Wondji *et al.*, 2009). The future availability of the full genome of *Ae. albopictus* will help to conclusively address this issue. However, the involvement of the CYP6P4 ortholog in permethrin resistance in *Ae. albopictus* will be similar to recent reports that CYP6P4 was involved in permethrin resistance in a population of the malaria vector *An. arabiensis* in Chad (Witzig *et al.*, 2013). It has also been associated with pyrethroid resistance in *An. gambiae* (Tene *et al.*, 2013) and in *An. funestus* (Wondji et al., 2009, Riveron et al., 2013). The likely involvement of the CYP6P4 ortholog in the permethrin resistance in the KL population of *Ae. albopictus* is further supported by the lower expression of this gene in the C-S comparison while it is highly over-expressed in the R-C and R-S comparison. In addition, the lower expression of the CYP6N3 gene in the R-C comparison in KL in contrast to the C-S comparison, it further supports that CYP6N3 is less involved in permethrin resistance but more likely plays a role in the bendiocarb resistance. Future functional analyses will confirm these observations. No CYP9 P450 was up-regulated in the R-C comparison further supporting that genes form this family contrary to *Ae. aegypti* which play no or little role in pyrethroid resistance in *Ae. albopictus*. The over-expression of genes belonging to other gene families such as glutathione-s-transferase, aldehyde oxidase, heat shock protein or short chain dehydrogenases has been commonly reported in other studies on mechanisms of metabolic resistance in various insects (Bariami et al., 2012, Vontas et al., 2005, Riveron et al., 2013, Kwiatkowska et al., 2013).

Conclusion

From this study, resistance in *Ae. albopictus* population in Malaysia could possibly be due to metabolic resistance involving detoxification genes mainly cytochrome P450s. However, to pinpoint the exact gene involved in which population and to resistance in specific insecticide further investigations needed to be done to further characterise the resistance mechanism. There seems to be no *kdr* mutations involved in the pyrethroid or DDT resistance in Malaysia but a mechanism of cuticular thickening in the *Ae. albopictus* could be a cause for resistance.

Control of *Ae. albopictus* is still effective by using pyrethroids as they are still susceptible to this insecticide in most populations. However, the presence of resistance in KL though cytochrome P450 up-regulation should be a concern and suitable resistance management strategies using the information on resistance mechanisms generated by this study should be implemented across Malaysia before the issue of resistance becomes worse and leads to control intervention failure for pyrethroid-based interventions.

6.0 GENERAL DISCUSSION AND CONCLUSION

The first report of dengue fever in Malaysia was in 1902 in Penang and the emergence of DHF was in 1962 (Ang and Satwant, 2001). Recently an increase in the number of dengue cases and related deaths has caused concern among the community. It was reported that 10,712 cases and 19 deaths have been recorded this year (2014) up to 6th February compared to only 2,836 cases with eight deaths over the same period in 2013 (Loh *et al.*, 2013). This was due to a new serotype of the dengue virus (DEN-2) which was previously only reported to be found in Singapore and spread to Malaysia at the end of 2013. Previously only DEN-4 serotype was present in Malaysia and the change of the serotype has caused different immune response to the dengue virus which led to the increase of cases (Loh *et al.*, 2013). Other than the presence of new dengue virus serotype, rapid industrial and economic growth which produced infrastructures and active construction sectors caused a substantial increase in man-made mosquito breeding sites. In addition, rural to urban migrations resulting in settlements with poor sanitation systems plus abundant tropical rainfall provide a haven for *Aedes* breeding (Ang and Satwant, 2001).

Since no anti-viral vaccination is present for the treatment of dengue virus, the only means of controlling the transmission of the disease is to control the abundance of the dengue vector populations, and the method employed by the Ministry of Health (MOH) in Malaysia is by using insecticides. Usually insecticides are not used routinely, they are only used during disease outbreaks and to eliminate adult vector mosquitoes (Chen *et al.*, 2006). Fogging is conducted within a radius of 200 – 400m from where a dengue case is reported, and repeat fogging is conducted 7 to 10 days after the first fogging. The insecticides commonly used for fogging are pyrethroids (Loke *et al.*, 2010). Larviciding by the use of temephos (Abate®) and *Bacillus thuringiensis israelensis* (Bti) have also been employed by the MOH to control the number of dengue cases (Tan *et al.*, 2012). The over reliance on the same insecticide class has caused insecticide resistance to develop in the dengue vectors.

For a successful vector control program, resistance management has to be considered by using insecticides more efficiently. To increase the efficacy of a compound, potent insecticides need to be used with a thorough understanding of the resistance profile present in the target organisms. At the present, there are a limited number of insecticides that have been licensed for the use in public health and no new insecticide have been developed for the control of adult mosquitoes for over 30 years (Hemingway *et al.*, 2006). This further strengthens the need to monitor insecticide resistance in a population and also understanding the underlying resistance mechanism.

The target site resistance is a resistance mechanism that has been reported around the world in various mosquito species and it is reasonably well understood. When a resistant allele is detected, a new tool could be developed for the screening of that mutation; such as the screening of *kdr* F1534C mutation in *Ae. aegypti* (Yanola *et al.*, 2011). However, the possibility of detecting new target site mutations in addition to those commonly observed should not be underestimated since the development of new mutations could be detrimental to vector control programs. An example is the recent discovery of a mutation in the A*n. gambiae* VGSC, the N1575Y mutation (Jones *et al.*, 2012).

Metabolic resistance has also been reported in various mosquito species but it is a more complex system (Ranson *et al.*, 2011). This is because there are multiple genes that could be involved in metabolic resistance and different molecular mechanisms for the metabolic resistance to occur (Ranson et al., 2011, Wilding et al., 2012). There could be mutations modifying the structure of the genes encoding specific detoxification enzymes which play a role in enhancing insecticide metabolism if they increase the specificity of the enzyme to a certain insecticide, such as glutathione-s-transferase GSTe2 in *An. funestus* (Riveron *et al.*, 2014). Another metabolic resistance mechanism which is more common is the increase in expression levels of detoxification genes which has been reported among others by Djouaka *et al.* (2008), Strode *et al.* (2008), Wondji *et al.* (2009) and Bariami *et al.* (2012).

Insecticide resistance has become an important issue in vector control programs worldwide and specifically in Malaysia. To date, no comprehensive study has been done to access the resistance profile of dengue vectors across Malaysia namely *Ae. aegypti* and *Ae. albopictus*. Insecticide susceptibility assays have been conducted and reported in only a few places in Malaysia such as in Kuala Lumpur since the main research institute the Institute for Medical Research (IMR) is located there and in Penang, where numerous work on the subject have been conducted at the Universiti Sains Malaysia (USM). But, no molecular characterisation of underlying insecticide resistance mechanism has been conducted.

This study was conducted to obtain a resistance profile of both *Aedes* species against various groups of insecticides across Malaysia as well as understanding the mechanisms that could potentially be responsible for resistance. The collection of the field samples were conducted in four states across Malaysia in 2010 to obtain an overall picture of the resistance present in *Ae. aegypti* and *Ae. albopictus* in Penang (PG), Kuala Lumpur (KL), Johor Bharu (JB) and Kota Bharu (KB). For larval bioassays, Ae*. albopictus* was slightly more resistant to temephos compared to *Ae. aegypti* with a higher RR value. Adult bioassays showed that *Ae. aegypti* was far more resistant towards pyrethroids (permethrin and deltamethrin) compared with *Ae. albopictus*. High level of DDT and bendiocarb resistance was observed in both *Aedes* species. *Aedes aegypti* was more susceptible towards malathion compared to *Ae. albopictus* showing total susceptibility in all the locations except for KL (91% mortality) and JB (99% mortality). It was also true for dieldrin resistance with *Ae. albopictus* showing a higher level of resistance when compared to *Ae. aegypti*.

The difference in susceptibility patterns between the two species across Malaysia demonstrate the importance of understanding the resistance mechanisms that could be involved. A summary of the resistance mechanisms observed in the two Malaysian dengue vectors are presented in Table 6.1.

Species	Target site mutations	Candidate genes implicated in metabolic resistance
Aedes aegypti	F1534C, V1016G	CYP9J27, CYP9M4, CYP9J26-609 (AAEL014609-RA), CYP9J26-607 (AAEL014607-RA), Trypsin
Aedes albopictus	None	CYP6N3, CYP6AE1, CYP6M2, CYP6P12, CYP9J17, GSTT3, GSTD1, ABCA

Table 6.1 Summary of resistance mechanisms detected in Malaysian populations of *Aedes aegypti* and *Aedes albopictus* in this study.

Once the resistance profile has been established, the samples were subjected to target-site mutation screening by pyrosequencing genotyping of the three commonly detected mutations which have been reported to confer resistance in *Ae. aegypti* (Saavedra-Rodriguez *et al*., 2007, Harris *et al.*, 2010). F1534C mutation was successfully observed in *Ae. aegypti* but not in *Ae. albopictus* . The sequencing of cDNA of exon19 to 31 of the VGSC gene additionally detected the V1016G mutation in *Ae. aegypti* but not in *Ae. albopictus.* The combined presence of target-site resistance through *kdr* mutations and metabolic resistance in *Ae. aegypti* could explain the higher level of resistance to pyrethroids observed in this species in contrast to *Ae. albopictus* where only the metabolic resistance is present. *Kdr* mutations have been reported in other *Ae. aegypti* strains but only a Singaporean strain of *Ae. albopictus* has been reported to have the F1534C mutation (Kasai *et al.*, 2011). The differences in the *kdr* mutation between the two species could be due to the nature of the breeding habitats of the species and the exposure to insecticides. *Aedes aegypti* is reported to be an urban species which breed and rests in close proximity to human dwellings. Hence, this species is regularly exposed to frequent usage of household insecticides which are mostly pyrethroid based. This caused *Ae. aegypti* to be more resistant towards pyrethroids and may have led to the selection of *kdr* mutations associated with pyrethroid resistance in contrast to *Ae.*

albopictus which is not expose to the same level of selection in its more rural settings.

Other target site resistance were also been screened for such as the sequencing of the Acetylcholinesterase (*Ace-1*) gene which could harbour mutations conferring resistance against carbamates and organophosphates. However, the experiment failed due to overlapping peaks in the sequences of both *Aedes* species. Further investigation has to be conducted by cloning the sequences. However, because this mutation has not been observed in *Aedes* mosquitoes (Vontas *et al.*, 2012) and that there was no consistent cross-resistance between carbamates and organophospates, it is likely that this mechanism also plays little role in Malaysia. RDL mutation is another target site resistance that could be studied in the Malaysian *Aedes* species especially as there was dieldrin resistance in the Johor Bharu *Ae. aegypti* populations and all the *Ae. albopictus* populations.

Genome-wide microarray-based analysis showed that metabolic resistance plays an important role in conferring resistance to insecticides in *Ae. aegypti* and *Ae. albopictus* across Malaysia. Over-expression of many genes belonging to the detoxification gene families especially the cytochrome P450 genes in PG, KL, KB (for *Ae. aegypti*) and JB (for *Ae. albopictus*) when comparing them to the laboratory susceptible strain supports the involvement of metabolic resistance mechanism in these species. Several P450 genes belonging to the CYP9 family were over-expressed in the C-S comparison in *Ae. aegypti* such as two transcripts of CYP9J26, CYP9J27, CYP9J28, CYP9M6 while only a few cytochrome P450s from the CYP6 family (CYP6P12, CYP6BB2) were over-expressed and usually at lower fold change. However, this was different for *Ae. albopictus* where more P450 genes belonging to the CYP6 family were over-expressed in the C-S comparison including CYP6N3, CYP6P12, CYP6Z6 CYP6AG6 while only few cytochrome P450s from the CYP9 family were over-expressed.

The involvement of the CYP9 family in *Ae. aegypti* were previously reported around the world (Strode *et al.*, 2008, Marcombe *et al.*, 2009, Bariami *et al.*, 2012, Vontas *et al.*, 2012). The involvement of CYP6 family in insecticide resistance in *Ae. albopictus* is rather similar to *Anopheles* mosquitoes (David et al., 2005, Muller et al., 2008b, Wondji et al., 2009, Riveron et al., 2013). This difference between *Ae. albopictus* and *Ae. aegypti* suggests that the speciation between these two species are extensive and have led to significant differences in their metabolic resistance mechanisms. *Aedes albopictus* in Malaysia is more similar to that of the malaria vector such as no *kdr* mutation has been detected and CYP6 gene family is more involved in the metabolic resistance. Overall, this study has highlighted that although *Ae. aegypti* and *Ae. albopictus* are both *Aedes* species which are vectors of dengue, they exhibit significant differences in the resistance profile, resistance mechanisms involved and also their ecology. This suggests that the speciation between these two dengue vectors could be extensive and what is true for one is not necessary the case for the other. Therefore, it is likely that further significant differences could be observed between the two species in various aspects of their biology, ecology, behaviour and role in disease transmission. For example, this study clearly suggests that the same control interventions might not work against both species due to their differences in resistance profiles and underlying resistance mechanisms.

Conclusion

This research is the first study to establish the resistance profile of *Ae. aegypti* and *Ae. albopictus* across Malaysia. It is also the first study that has recorded the presence of specific target site mutations and metabolic resistance mechanisms at the molecular level in both dengue vectors. The differences in the resistance mechanisms in both species as well as the different locations could give us an opportunity to design a control program which best suits the surrounding. For example, to implement integrated vector management by combining source reduction and the use of insecticides to control the dengue vectors. Suggestions

could be made to the MOH Malaysia regarding the best insecticides to be used in specific regions depending on the resistance profile such as using malathion for ULV and thermal space spraying rather than permethrin in locations where high level of permethrin resistance was recorded.

However, with resistance threatening the control programs in Malaysia, the use of alternative vector control methods such as public awareness and larval source reduction should be considered. As mentioned in Chapter 1, the ’10 minutes a day’ campaign conducted by MOH Malaysia was not successful due to the lack of cooperation from the public (Wan-Norafikah *et al.*, 2010). With proper education, this campaign could be made successful since most people do not understand the life cycle of the dengue vector and importance of removing breeding sites. Community participation and elimination of potential breeding sites are important aspects of a successful vector control program as reported by Pilger *et al.* (2009) and Horstick *et al.*(2010). Biological control by using natural enemies of the dengue vector and also sterile insect techniques such as the release of mosquitoes carrying a dominant lethal (RIDL) gene which was conducted in the Cayman Islands (Harris *et al.*, 2010) could also be done.

Regular monitoring and evaluation of dengue vector populations is also important in ensuring a successful and sustainable vector control program (Horstick et al., 2010). It is important to conduct regular assessment of resistance status in a certain location because it is essential to understand and manage insecticide resistance to prevent the spread of resistance which could lead to failure of control programs and increase in the number of dengue cases.

To strengthen or add to this research, functional characterisation of the candidate genes obtained from the microarray experiment could be conducted to confirm the involvement of the genes in the detoxification of the insecticides. Further work in identifying the target site mutation could also be done such as conducting an assay to identify mutation that is responsible for the dieldrin resistance (resistance in the GABA receptor). New techniques to analyse the expression profiles could also be used such as the RNA sequencing and exome sequencing

techniques to further assess the main resistance genes and detect resistance markers which could be used to design field applicable molecular diagnostic assays for early detection of resistance in the field.

In conclusion, besides the use of insecticides coupled with a continuous assessment of resistance, sustainable methods for dengue control should also be conducted. Sanitation in Malaysia is not at its' best, therefore strict regulations in source reduction should be implemented to reduce the breeding sites of the *Aedes* mosquitoes. Public awareness is also important since without the help of the public, proper source reduction management will not succeed. It is understood that there is no quick an easy way to eliminate the spread of dengue cases, but with proper planning and a sustainable, cost effective and integrated vector control approach the number of dengue cases could potentially be substantially reduced in Malaysia.

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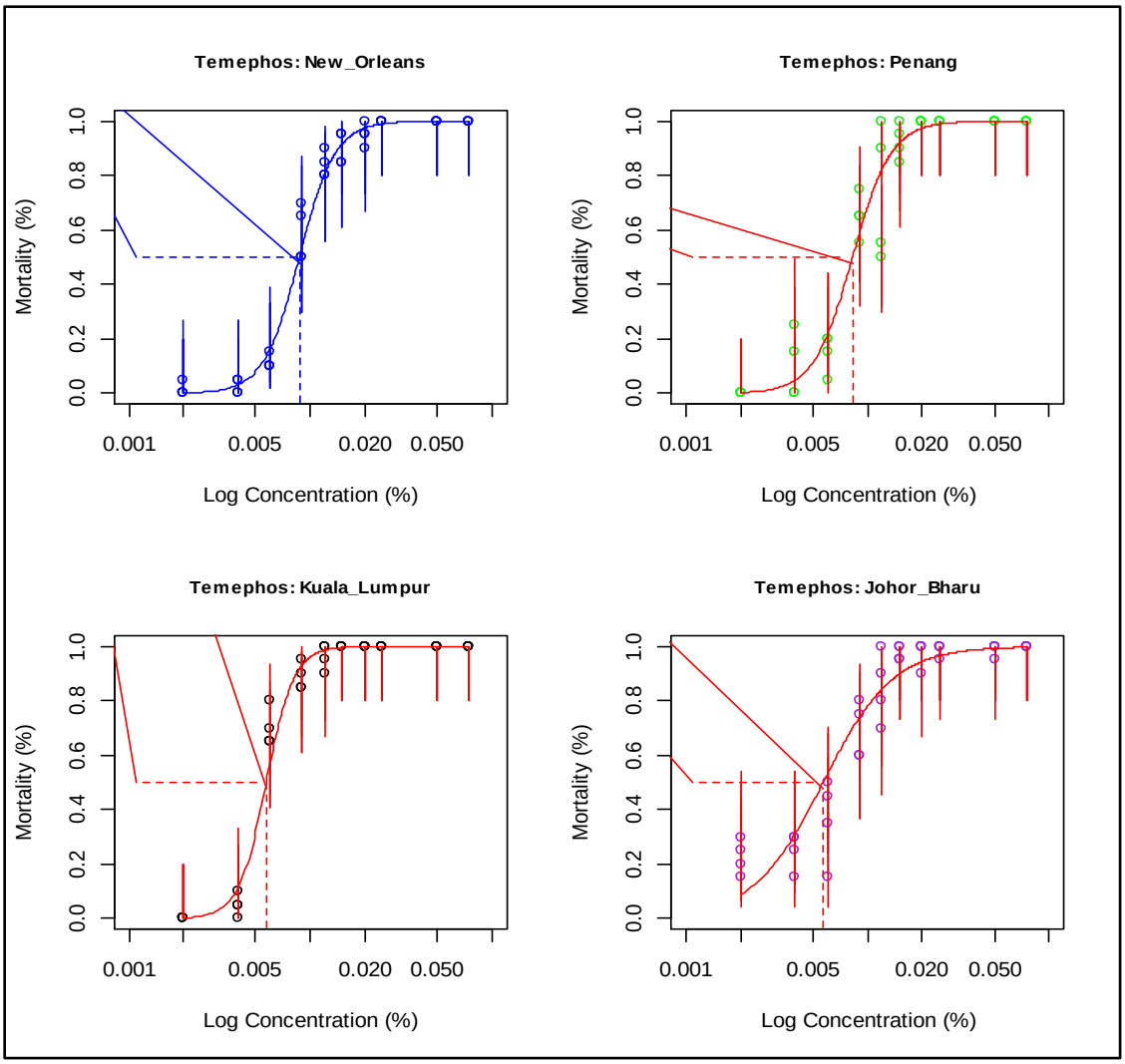
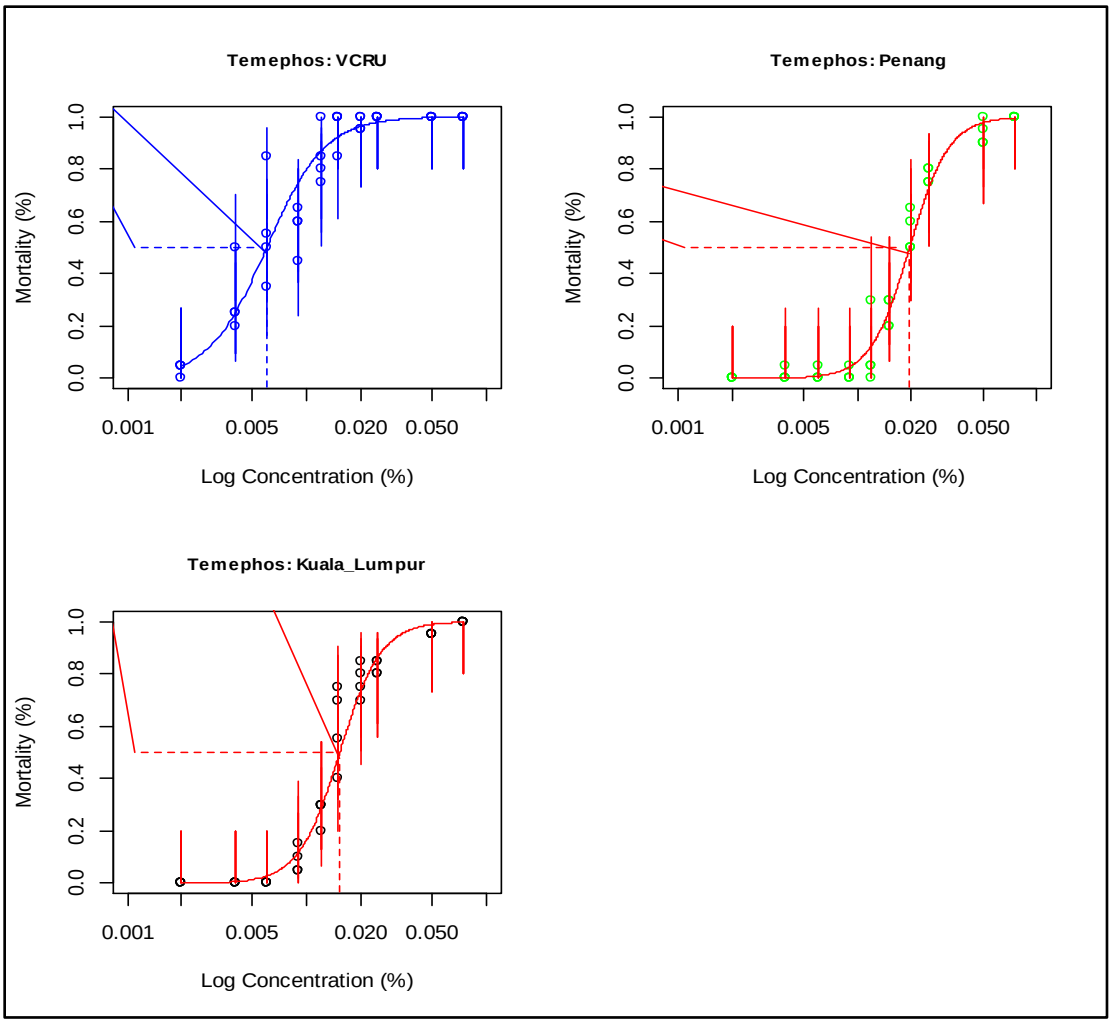
8.0 APPENDICES

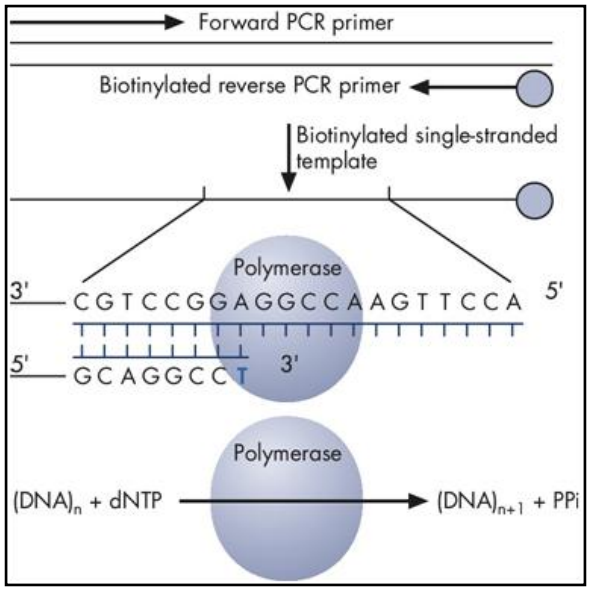
Appendix 8.1 Probit graph for LC_{50} of different strains of *Ae. aegypti.*



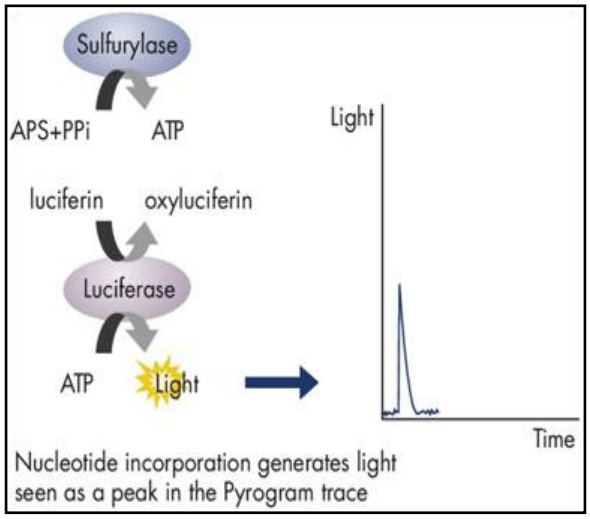
Appendix 8.2 Probit graph for LC_{50} of different strains of *Ae. albopictus* .



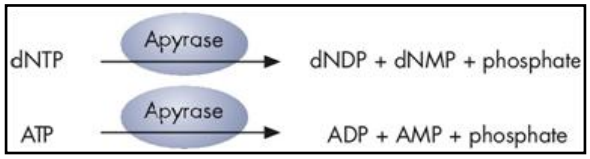
Appendix 8.3 Principle of Pyrosequencing. (taken from www.qiagen.com)



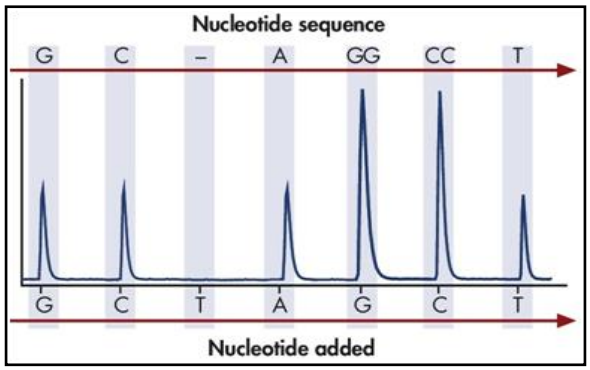
[1] A DNA segment is amplified and the strand to serve as the Pyrosequencing template is biotinylated. After denaturation, the biotinylated single-stranded PCR amplicon is isolated and allowed to hybridize with a sequencing primer. [2] The hybridized primer and single-stranded template are incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, as well as the substrates adenosine 5' phosphosulfate (APS) and luciferin. [3] The first deoxyribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyzes addition of the dNTP to the sequencing primer, if it is complementary to the base in the template strand. Each incorporation event is accompanied by the release of pyrophosphate (PPi) in a quantity equimolar to



[4] ATP sulfurylase converts PPi to ATP in the presence of APS. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by CCD sensors and seen as a peak in the raw data output (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated.



[5] Apyrase continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is



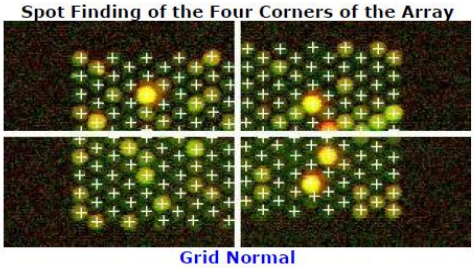
[6] Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alfa-thio triphosphate (dATPαS) is used as a substitute for natural deoxyadenosine triphosphate (dATP) since it is efficiently used by DNA polymerase, but not recognized by luciferase. As the process continues, the complementary DNA strand is elongated and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.

Appendix 8.4 Example of QC report from microarray hybridisation.

QC Report - Agilent Technologies : 2 Color Gene Expression — QCMetrics InRange (11 of 11)

Date	Wednesday, November 14, 2012 - 14:46	BG Method	No Background
Image	US84700254_253938210004_S01_H [1_4]	Background Detrend	On(FeatNCRange, LoPass)
Protocol	GE2_105_Jan09 (Read Only)	Multiplicative Detrend	True
User Name	Administrator	Dye Norm	Linear Lowess
Grid	039382_D_F_20120306	Linear DyeNorm Factor	3.16(Red) 3.52(Green)
FE Version	10.5.1.1	Additive Error	13(Red)13(Green)
Sample(red/green)		Saturation Value	661870 (r), 651537 (g)

Spot Finding of the Four Corners of the Array

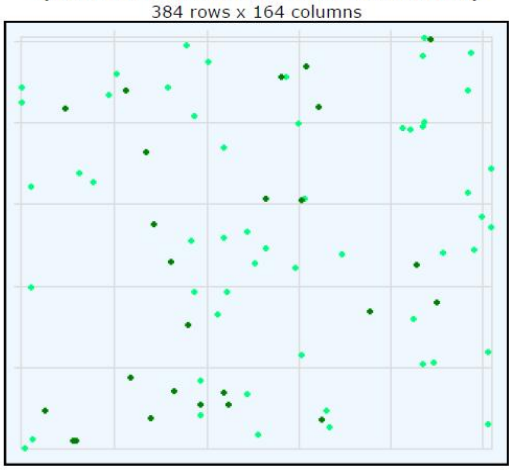


Grid Normal

	Feature		Local Background	
	Red	Green	Red	Green
Non Uniform	25	25	0	0
Population	73	57	475	0

Spatial Distribution of All Outliers on the Array

384 rows x 164 columns



Net Signal Statistics

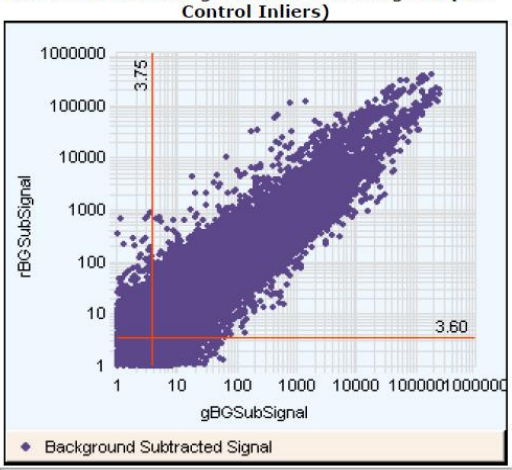
Agilent SpikeIns:

	Red	Green
# Saturated Features	0	0
99% of Sig. Distrib.	24726	22137
50% of Sig. Distrib.	3120	2842
1% of Sig. Distrib.	183	159

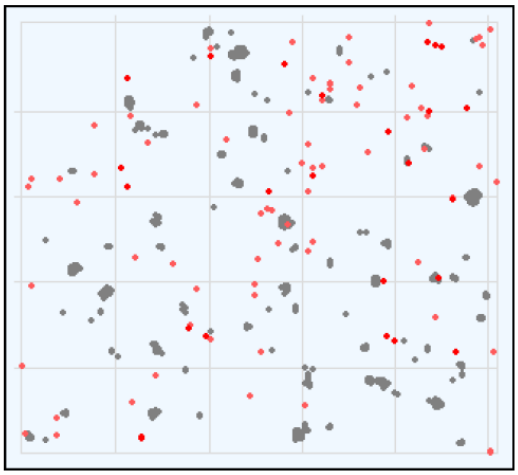
Non-Control probes:

	Red	Green
# Saturated Features	0	0
99% of Sig. Distrib.	60202	20583
50% of Sig. Distrib.	52	44
1% of Sig. Distrib.	40	32

Red and Green Background Corrected Signals (Non-Control Inliers)



Features (NonCtrl) with BGSubSignals < 0: 20615 (Red); 18699 (Green)



FeatureNonUnif (Red or Green) = 46(0.07%)

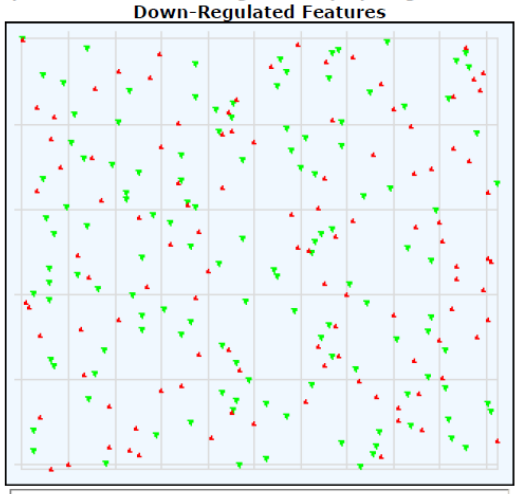
GeneNonUnif (Red or Green) = 22 (0.037 %)

BG NonUniform BG Population

Red FeaturePopulation Red Feature NonUniform

Green FeaturePopulation Green Feature NonUniform

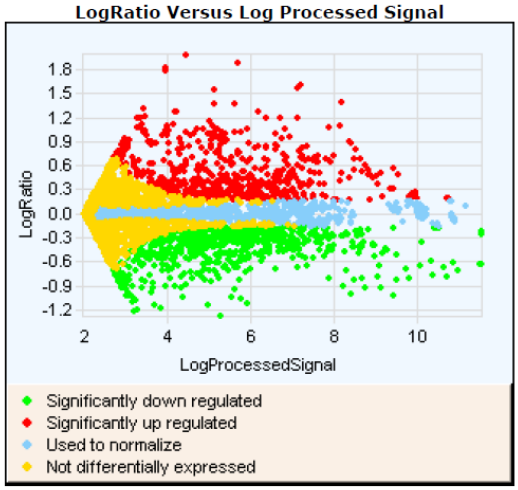
Spatial Distribution of Significantly Up-Regulated and Down-Regulated Features



#Up-Regulated:5368 (Red) ; #Down-Regulated:6309 (Green)

Up-Regulated Down-Regulated

LogRatio Versus Log Processed Signal



Negative Control Stats

	Red	Green
Average Net Signals	45.85	38.22
StdDev Net Signals	3.12	2.92
Average BG Sub Signal	-2.71	-1.81
StdDev BG Sub Signal	3.05	2.78

Local Bkg (inliers)

	Red	Green
Number	62501	62976
Avg	49.27	28.45
SD	1.82	2.60

Foreground Surface Fit

	Red	Green
RMS_Fit	1.22	1.43
RMS_Resid	4.00	3.72
Avg_Fit	55.78	47.23

Reproducibility: %CV for Replicated Probes

	Median %CV Signal (inliers)			
	Non-Control probes		Agilent SpikeIns	
	Red	Green	Red	Green
BGSubSignal	9.36	9.94	11.08	11.81
ProcessedSignal	6.67	7.39	5.47	9.16

Array Uniformity: LogRatios

	Non-Control	Agilent SpikeIns
AbsAvgLogRatio	0.39	0.50
AverageS/N	7.14	18.98

Sensitivity:Agilent SpikeIns - Ratio of Signal to Background for 2 dimmest probes

(+)E1A_r60_n11		(+)E1A_r60_a97	
(g)	**(r)**	**(g)**	**(r)**
4.0	13.6	10.1	5.1

Agilent SpikeIns Signal Statistics

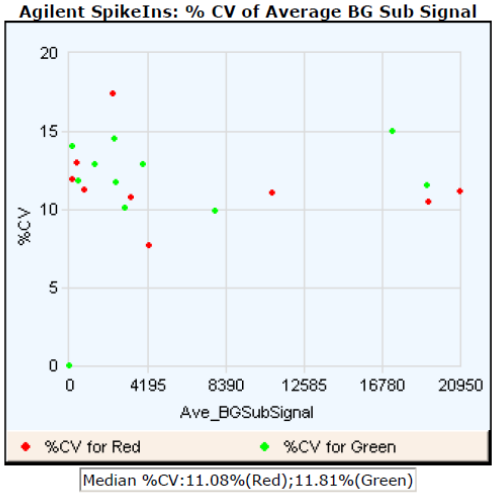
Probe Name	Exp	Obs	SD	S/N
(+)E1A_r60_n9	-1.00	-0.92	0.02	40.93
(+)E1A_r60_a107	-0.48	-0.39	0.02	17.39
(+)E1A_r60_a135	-0.48	-0.34	0.02	18.05
(+)E1A_r60_n11	-0.48	-0.49	0.03	14.22
(+)E1A_r60_1	0.00	0.16	0.02	8.36
(+)E1A_r60_a20	0.00	-0.33	0.04	8.79
(+)E1A_r60_3	0.48	0.62	0.02	31.83
(+)E1A_r60_a104	0.48	0.37	0.03	13.93
(+)E1A_r60_a97	0.48	0.49	0.04	13.25
(+)E1A_r60_a22	1.00	0.85	0.04	23.06

Evaluation Metrics for GE2_QCMT_Jan09

Metric Name	Value	UpLim	LowLim	IsMandatory
AnyColorPrcntFeatNonUnif...	0.07	1.00	NA	False
absE1aObsVsExpCorr	0.94	NA	0.86	False
absE1aObsVsExpSlope	0.91	NA	0.85	False
gE1aMedCVBkSubSignal	11.81	25.00	NA	False
gNegCtrlAveBGSubSig	-1.81	10.00	-20.00	False
gNegCtrlSDevBGSubSig	2.78	15.00	NA	False
gNonCntrlMedCVBkSubSignal	9.94	25.00	NA	False
rE1aMedCVBkSubSignal	11.08	25.00	NA	False
rNegCtrlAveBGSubSig	-2.71	4.00	-20.00	False
rNegCtrlSDevBGSubSig	3.05	6.00	NA	False
rNonCntrlMedCVBkSubSignal	9.36	25.00	NA	False

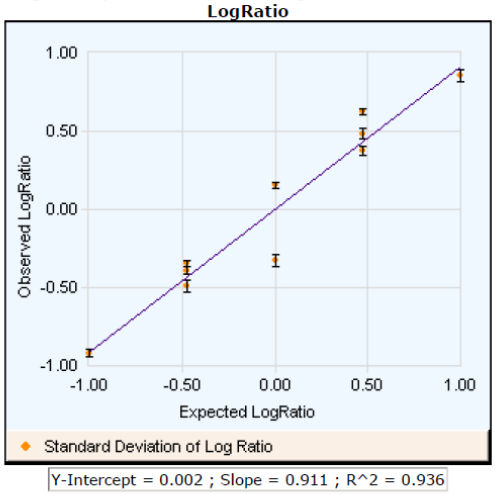
♦ In Normal Range ♦ Evaluate

Agilent SpikeIns: % CV of Average BG Sub Signal



Median %CV:11.08%(Red);11.81%(Green)

Agilent SpikeIns: Expected LogRatio Vs Observed LogRatio



Y-Intercept = 0.002 ; Slope = 0.911 ; R^2 = 0.936